

B205



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/17, 48/00, C07H 21/04, C07K 14/435, C12N 15/00, 15/63, 15/85, C12Q 1/68	A1	(11) International Publication Number: WO 99/02179 (43) International Publication Date: 21 January 1999 (21.01.99)
(21) International Application Number: PCT/US98/14414 (22) International Filing Date: 9 July 1998 (09.07.98) (30) Priority Data: 08/890,865 10 July 1997 (10.07.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/890,865 (CIP) Filed on 10 July 1997 (10.07.97) (71) Applicant (for all designated States except US): THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK [US/US]; West 116th Street and Broadway, New York, NY 10027 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CONSTANTINI, Franklin [US/US]; Apartment 4J, 1161 York Avenue, New York, NY 10021 (US). ZENG, Li [CN/US]; Apartment 7H, 100 Haven Avenue, New York, NY 10032 (US).		(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US). (81) Designated States: AU, CA, JP, MX, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: AXIN GENE AND USES THEREOF (57) Abstract <p>This invention provides an isolated nucleic acid which encodes Axin. This invention further provides an isolated nucleic acid which encodes a polypeptide comprising the amino acid sequence of Axin. This invention further provides a purified wildtype or mutant Axin. This invention further provides an oligonucleotide capable of distinguishing nucleic acids encoding mutant or wildtype Axin. This invention also provides various methods of use, such as a method for determining whether a subject carries a mutation in the Axin gene, a method of determining whether a subject has a predisposition for cancer, a method for treating a subject who has a predisposition to cancer, a method for determining whether a subject has cancer, a method for detecting a mutation in cancerous cells of the subject, a method of suppressing cells unable to regulate themselves and a method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves. This invention also provides a variety of pharmaceutical compositions and a method of treating a subject who has cancer comprising administration of the pharmaceutical compositions. This invention also provides a transgenic, nonhuman mammal, specifically a transgenic expressing mutant Axin.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Larvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

AXIN GENE AND USES THEREOF

5 This application claims priority of U.S. Serial No. 08/890,865, filed July 10, 1997, the contents of which are hereby incorporated by reference into the present application.

10 The invention disclosed herein was made with Government support under Grant No. DK-46934 from the National Institutes of Health of the United States Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

15 Throughout this application, various publications are referenced in parenthesis. Full citations for these publications may be found listed at the end of the specification. The disclosures of these publications in
20 their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein.

Introduction

25 A fundamental problem in mammalian embryology is the mechanism by which the egg cylinder, an epithelial cup in which only the dorsal-ventral axis is established, gives rise to an embryo with anterior-posterior (A-P) polarity. In
5 the mouse, the earliest morphological manifestation of the A-P axis is the delamination of mesoderm in the primitive streak at ~E6.5. The position of the streak cannot be predicted by earlier morphological asymmetries in the embryo (Gardner et al., 1992), and the regulative abilities of
10 early mouse embryos appear to rule out axis determination by localized determinants from the egg. While a few secreted factors or transcription factors are expressed asymmetrically in the egg cylinder shortly before primitive streak formation, and thus might be involved in induction of
15 the streak, their roles in this process have not been

-2-

established (reviewed by Bachvarova, 1996; Conlon and Beddington, 1995).

5 In the amphibian embryo, the dorsal-ventral axis (the second axis to be specified, analogous to the A-P axis of the mouse) is determined by the point of sperm entry and subsequent cortical rotation. This rotation generates the Nieuwkoop Center, a group of dorsal/vegetal blastomeres that induce formation of the Spemann organizer. Transplantation
10 of the Nieuwkoop Center or the organizer to an ectopic position induces the formation of a complete secondary axis, i.e., notochord, somites, neural tube, and head structures (reviewed by Slack, 1994). Recent studies suggest that the formation of the Nieuwkoop Center depends on activation of
15 components of the Wnt signaling pathway (Carnac et al., 1996; Fagotto et al., 1997; Wylie et al., 1996). The Wnts are a family of secreted polypeptides related to *Drosophila* wingless, whose receptors are believed to be members of the frizzled family (reviewed by Miller and Moon, 1996). The
20 next known component of the signaling pathway is Dishevelled (Dsh), a cytoplasmic protein that, when activated by a Wnt signal, inhibits the activity of glycogen synthase kinase-3 (GSK-3). In the absence of a Wnt signal, GSK-3 activity leads (directly or indirectly) to the phosphorylation and
25 consequent degradation of β -catenin. In the presence of a Wnt signal, GSK-3 is inhibited, increasing the cytosolic level of β -catenin, and promoting its interaction with downstream effectors.

30 A role for the Wnt signaling pathway in development of the amphibian embryonic axis was revealed by the ability of several Wnts, or downstream factors, to induce an ectopic axis when injected into *Xenopus* embryos (Miller and Moon, 1996). Furthermore, components of this pathway are required
35 for normal axial development because depletion of maternal β -catenin mRNA (Heasman et al., 1994), or sequestration of β -catenin to the plasma membrane (Fagotto et al., 1996), results in ventralized embryos that fail to develop a dorsal

axis. However, it is not clear whether a Wnt ligand triggers Nieuwkoop Center formation, or whether downstream components of the Wnt pathway are activated by some other mechanism (Hoppler et al., 1996; Miller and Moon, 1996; Sokol, 1996).
5 The Nieuwkoop Center is thought to induce a Spemann organizer by secreting a (yet to be identified) diffusible signal (Fagotto et al., 1997; Wylie et al., 1996), which may act synergistically with mesoderm-inducing factors, such as Activin and Vg1, to activate the expression of dorsal-specific genes, such as Goosecoid (Watabe et al., 1995).
10 Dorsoventral patterning of the mesoderm is further controlled by opposing signals emanating from the organizer and the ventral mesoderm: a ventral bone morphogenetic protein (BMP) signal represses dorsal genes, while in the dorsal side the secreted factors Noggin, Chordin and
15 Follistatin directly inhibit BMPs (Hogan, 1996).

While little is known about the molecular control of axis formation in mammalian embryos, a potential source of
20 insight is the study of mouse mutants that affect this process (Conlon and Beddington, 1995; St-Jacques and McMahon, 1996), such as *Fused* (*Fu*). Two spontaneous alleles of *Fu*, called *Kinky* (*Fu^{Ki}*) and *Knobbly* (*Fu^{Kb}*), and a transgenic insertional allele, *Fu^{Ty1}* (previously called
25 *He46*), carry recessive mutations that are lethal at E8-E10 (Gluecksohn-Schoenheimer, 1949; Jacobs-Cohen et al., 1984; Perry et al., 1995). In addition to neuroectodermal and cardiac abnormalities, a remarkable property of many early post-implantation embryos homozygous for these three mutant
30 alleles is a duplication of the embryonic axis. This phenotype, unique among mouse mutants, led nearly 40 years ago to the suggestion that *Fu* may play a role in the specification of the embryonic axis (Gluecksohn-Schoenheimer, 1949). *Kinky*, *Knobbly* and a third spontaneous
35 allele, *Fused* (*Fu^{Fu}*), but not *Fu^{Ty1}*, also have dominant effects that include transient bifurcations of the fetal tailbud, asymmetric fusion of vertebrae leading to tail kinks, deafness, and neurological defects (Lyon et al.,

1996).

The cloning of this locus with the aid of a transgene insertion was previously described (Perry et al., 1995).
5 Here, the isolation and sequence of cDNA clones, and the genomic structure of the wild type (WT) and *Fu^{Tg1}* alleles are reported. Analysis of the *Fu^{Fu}* and *Fu^{Kb}* alleles (Vasicek et al., manuscript submitted) has shown that both are caused by retroviral insertions. Because two mutant alleles causing
10 axial duplications in homozygous embryos, *Fu^{Tg1}* and *Fu^{Kb}*, disrupt production of the major mRNA, the normal gene product may negatively regulate a critical step in the formation of the embryonic axis. This hypothesis is supported by studies in *Xenopus* embryos, which demonstrate
15 that dorsal injection of WT *Fused* mRNA blocks axis formation, while ventral injection of a dominant-negative mutant form induces an ectopic axis. Co-injection with factors acting at various steps in axis formation reveals that *Fused* exerts its effects at a very early stage, by
20 specifically inhibiting signal transduction through the Wnt pathway in the Nieuwkoop Center. Thus, analysis of the *Fu* locus has identified a novel inhibitor of the Wnt signaling pathway, and suggests that the same pathway regulates an early step in embryonic axis formation in mammals and
25 amphibians. To avoid confusion with the unrelated *Drosophila* gene *fused*, applicants have renamed the *Fu* gene "Axin", for "axis inhibition".

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid which encodes Axin.

5

This invention also provides an isolated nucleic acid which encodes a polypeptide comprising the amino acid sequence of Axin.

10 This invention also provides a polypeptide comprising the amino acid sequence of Axin. This invention also provides a purified wildtype Axin or purified mutant Axin.

15 This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes wildtype Axin without hybridizing to a nucleic acid which encodes mutant Axin, and an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing
20 with a sequence of nucleotides present within the nucleic acid which encodes mutant Axin without hybridizing to a nucleic acid which encodes wildtype Axin.

25 This invention also provides a method for determining whether a subject carries a mutation in the axin gene which comprises (a) obtaining an appropriate nucleic acid sample from the subject and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine
30 whether a subject carries a mutation in the axin gene.

This invention further provides a method for determining whether a subject has a predisposition for cancer which comprises (a) obtaining an appropriate nucleic acid sample
35 from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has a predisposition for cancer.

-6-

This invention further provides a method for treating a subject who has a predisposition to cancer by either introducing the isolated nucleic acid encoding the wildtype Axin or an effective amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby treat the subject who is susceptible to cancer.

This invention also provides a method for determining whether a subject has cancer, which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

This invention also provides a method for detecting a mutation in cancerous cells of the subject which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

This invention further provides a method of suppressing cells unable to regulate themselves by either introducing the isolated nucleic acid encoding wildtype Axin or wildtype Axin in an amount effective enough to suppress the cells.

This invention further provides a method of treating a subject who has cancer by either introducing the isolated nucleic acid encoding wildtype Axin or the protein itself in an effective amount.

This invention further provides a method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises (a) contacting mutant Axin with the chemical compound under conditions permitting binding between the mutant Axin and

-7-

the chemical compound; (b) detecting specific binding of the chemical compound to the mutant Axin; and (c) determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.

This invention further provides pharmaceutical composition comprising a chemical compound capable of inhibiting cancer, an antisense molecule capable of inhibiting an isolated nucleic acid encoding mutant Axin, or purified Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

This invention further provides a method of treating a subject who has cancer comprising administration of an effective amount of the above-identified pharmaceutical composition

This invention further provides transgenic, nonhuman mammal comprising an isolated nucleic acid encoding Axin, specifically the mutant protein.

BRIEF DESCRIPTION OF THE FIGURES

Abbreviations: The amino acid residues are abbreviated as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. CON: consensus sequence.

Figure 1. Structure of the WT *Axin* gene and the transgenic allele *Axin^{Ty1}*. In *Axin^{Ty1}*, a random transgene insertion (Perry et al., 1995) was accompanied by a deletion including exon 2. Exon 1 is located in a CpG island, as indicated by the frequency of CpG or GpC dinucleotides per 100 bp, and the ratio of CpG/GpC, calculated at 50 bp intervals (left inset). A genomic probe used to isolate cDNA clones is indicated below the inset. The open box in exon 10 represents the 3' UTR. The inset at right shows the origin of form 1 and 2 alternatively spliced mRNAs. The cDNA sequences corresponding to exon 1 are 1-308; exon 2, 309-1267; exon 3, 1268-1408; exon 4, 1409-1505; exon 5, 1506-1643; exon 6, 1644-2161; exon 7, 2162-2332; exon 8, 2333-2578; exon 8A, 2579-2686; exon 9, 2687-2854; and exon 10, 2855-3731. The RGS region is encoded in exon 2, and the Dsh homology in exons 9-10. Restriction sites: N, NotI; S, SacII; X, XbaI; B, BamHI.

Figures 2A-2C. Expression of *Axin* mRNA in adult tissues, embryos and ES cells.

- 2A. Northern blot. ES cells are WT (+/+) or *Axin^{Ty1/Ty1}* (-/-). Sg, salivary gland; Th, thymus; Te, testis; Lu, lung; He, heart; Ki, kidney; Br, brain; Ov, ovary; Sp, spleen; Li, liver.
- 2B. Whole mount *in situ* hybridization analysis of *Axin* mRNA in (left to right) two E7.5, two E8.5 and one E9.5 WT embryos. Scale bar, 0.2mm.
- 2C. Expression of both mRNA isoforms in tissues and ES cells, detected by RT-PCR using primers flanking the 108 bp sequence encoded by exon 8A. The upper band (563

-9-

bp) represents form 2 mRNA and the lower band (455 bp) form 1. C, control with no added cDNA. m, 123 bp ladder.

5 Figures 3A-3D. Amino acid sequence of mouse Axin and its human and chicken homologs, and similarity to RGS and Dsh proteins.

3A-3B. Mouse (m), human (h) and chick (c) Axin sequences. Identical residues are highlighted in black, blue or red and conserved residues in gray. RGS and Dsh homologies are highlighted in blue and red, respectively. The mouse sequence begins with the first residue encoded by the cDNA sequence, and the first two Met residues are indicated by *.
 10 Also indicated is a 36 aa segment included in murine and human form 2. The mouse and human form 1 and the chicken sequence contain a bipartite NLS consensus at position 749 (K/R, K/R, 10 aa spacer, followed by 3 K/R in the next 5 residues), which is interrupted in form 2. In addition, mAxin includes a second NLS at aa 59. The murine sequence contains one consensus site for tyrosine phosphorylation (aa 192-199), and several for cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II, and GSK-3. The site of
 15 intron 6, where Axin^{Fu} contains a proviral insertion, is marked by a green triangle, and the site where exon 7 is interrupted in Axin^{Kb}, by a magenta triangle.

20 3C. Alignment of the RGS domains of Axin and 8 human or rat RGS proteins.

25 3D. Alignment of a 51 aa segment of Axin with a similar region in Drosophila Dsh and two murine homologs.

30 35 Figures 4A-4C. Dorsal injection of Axin mRNA ventralizes *Xenopus* embryos.

4A. Ventralization by dorsal injection of Axin, and rescue

-10-

- by β -catenin or *Siamois* but not *Xwnt8*. 2 ng of *Axin* mRNA, either alone or together with the other mRNA indicated, was injected into each of two dorsal blastomeres at the 4-cell stage. Embryos were evaluated at the tadpole stage (Table 1), and examples are shown. The amount of *Xwnt8* (20 pg), β -catenin (300 pg) or *Siamois* (100 pg) mRNA used was the minimal amount required to obtain full axis induction when each was injected alone in one ventral blastomere (see Figs. 5A & 5B). Scale bar, 1mm.
- 4B. Dorsal injection of *Axin* reduces expression of dorsal markers *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3*, but not the ubiquitously expressed elongation factor *EFL*. Each column shows the RT-PCR analysis of a pool of uninjected embryos or embryos injected at the 4-cell stage with *Axin* or control β -gal mRNA (2 ng), and grown to early gastrulae. -RT, control experiments in which RNA from uninjected embryos was processed without reverse transcriptase.
- 4C. Dorsal co-injection of β -catenin with *Axin* restores expression of *Siamois* and *Goosecoid*, and co-injection of *Siamois* restores *Goosecoid* expression, while co-injection of *Xwnt8* has no effect. Note that the injected *Siamois* (not detected with the primers used in this assay) does not induce expression of endogenous *Siamois*.

Figures 5A-5C. Ability of *Axin* to block ectopic axis formation.

- 5A. Ventral co-injection of *Axin* mRNA inhibits ectopic axis formation by upstream components of the Wnt pathway (*Xwnt8*, *Xdsh* and *dnGSK-3*), but not by β -catenin or *Siamois*, nor by *Activin*, *Noggin* or Δ *BMPR*. mRNA encoding the indicated dorsalizing factor was injected subequatorially in one ventral blastomere at the 4-8 cell stage, with or without 1 ng *Axin*, and embryos were examined for axial duplications at the late neurula - tailbud stage. The fraction of embryos with duplicated

-11-

- axes is indicated above each bar. mRNAs were injected in the minimal amounts needed to induce ectopic axes at high frequency: 10-20 pg *Xwnt8*, 1.5 ng *Xdsh*, 2 ng *dnGSK-3*, 300 pg β -catenin, 100 pg *Siamois*, 7.5 pg *Activin*, 200 pg *noggin*, or 1 ng Δ *BMPR* (Fagotto et al., 1997). *Activin*-induced secondary axes were generally very incomplete. Higher amounts of *Activin* mRNA lead to uninterpretable phenotypes.
- 5B. Examples of injected embryos. Scale bar, 2mm.
- 5C. Co-injection of *Axin* mRNA inhibits induction of the dorsal marker *Goosecoid* by *Xwnt8*, but not by *Activin*, *Noggin*, or Δ *BMPR*. Ectopic expression of *Goosecoid* in the ventral half of early gastrulae (stage 10 1/2) was analyzed by RT-PCR. Dorsal (D) and ventral (V) halves of uninjected embryos served as positive and negative controls (ctrl) for normal expression of *Goosecoid*.

Figures 6A-6G. Axis duplications in *Xenopus* embryos injected ventrally with Δ *RGs*, and in mouse embryos homozygous for the loss-of-function *Axin*^{Ty1} allele.

- 6A & 6B. *Xenopus* embryos with axis duplications caused by injection of 2 ng Δ *RGs* in one ventral blastomere (6A) or 1 ng Δ *RGs* in two ventral blastomeres (6B). The embryo in 6B is also strongly dorsalized. Scale bars, 0.5mm.
- 6C. Frequency of axis duplications in embryos injected with *Axin*, Δ *RGs*, or Δ *RGs* together with *Axin* or *C-cadherin*.
- 6D. Ectopic expression of dorsal markers in embryos injected ventrally with Δ *RGs*. Each column shows the RT-PCR analysis of the dorsal (D) or ventral (V) halves of a pool of embryos. In uninjected embryos, *Siamois*, *Goosecoid*, *Chordin*, and *Xnr3* are expressed dorsally. Ventral injection of Δ *RGs*, but not *Axin*, induces ectopic expression of the four dorsal markers.
- 6E-6G. Lateral view of a normal E7.5 mouse embryo (e6E) and two E8.5 *Axin*^{Ty1/Ty1} embryos with axial

-12-

5 duplications (6F and 6G), visualized by *in situ* hybridization to *HNF-3 β* , a marker of anterior axial mesoderm (Sasaki and Hogan, 1994). White arrows, primary axes; black arrowheads, ectopic axes. Scale bars, 0.2mm.

Figure 7. Model for the inhibitory effect of Axin on Wnt signal transduction. Established components of the Wnt pathway in the Nieuwkoop Center are indicated by blue symbols and solid black arrows, and positions where Axin might inhibit the pathway are indicated by red symbols and dashed arrows. GSK-3 promotes the degradation of β -catenin, while Wnt signals inhibit GSK-3 (via Dsh) and lead to accumulation of cytosolic β -catenin and expression of Siamois. Axin blocks the stimulation of this pathway by Wnt, Dsh or dominant-negative GSK-3, but not by overexpression of β -catenin or Siamois. Three alternative hypotheses are illustrated : (1) Axin might inhibit a protein phosphatase (PP2A) that may otherwise dephosphorylate substrates of GSK-3; (2) Axin might stimulate the activity of GSK-3 through an unknown mechanism; (3) Axin might inhibit, via its RGS domain, the transmission of a second signal (signal 2) involving a G-protein-coupled receptor, which would otherwise stimulate the Wnt pathway downstream of GSK-3. See text for further details.

Figure 8. Amino acid sequence of murine Axin (Sequence I.D. No. 1).

30 Figures 9A-9B. Nucleic acid sequence of murine Axin (Sequence I.D. No. 2).

Figures 10A-10B. Nucleic acid sequence of human Axin (Sequence I.D. No. 3).

35

Figure 11. Amino acid sequence of human Axin. (Sequence I.D. No. 4).

-13-

DETAILED DESCRIPTION

Throughout this application, references to specific nucleotides are to nucleotides present on the coding strand of the nucleic acid. The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

	C=cytosine	A=adenosine
10	T=thymidine	G=guanosine

A "gene" means a nucleic acid molecule, the sequence of which includes all the information required for the normal regulated production of a particular protein, including the structural coding sequence, promoters and enhancers.

The nucleic acids or oligonucleotides of the subject invention also include nucleic acids or oligonucleotides coding for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These nucleic acids or oligonucleotides include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The nucleic acids and oligonucleotides described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as

-14-

products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention provides for an isolated nucleic acid which encodes Axin. This isolated nucleic acid may be DNA or RNA, specifically cDNA or genomic DNA. This isolated nucleic acid also encodes mutant Axin or the wildtype protein.

Specifically, the isolated nucleic acid encodes a mouse wildtype Axin. This isolated nucleic acid may have the sequence designated Seq. I.D. No.: 2. Also, the isolated nucleic acid may have substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 1.

The isolated nucleic acid may also encode a human Axin having substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 4. Specifically the isolated nucleic acid has the sequence designated Seq. I.D. No.: 3.

This isolated nucleic acid may also encode a polypeptide comprising the amino acid sequence of Axin.

As used in this application, "Axin" means and includes any polypeptide having Axin activity, e.g. negative regulation of the Wnt signalling pathway, and having an amino acid sequence homologous to the amino acid sequence of mouse Axin (the sequence of which is set forth in Sequence I.D. No.: 1). Thus, this term includes any such polypeptide whether naturally occurring and obtained by purification from natural sources or non-naturally occurring and obtained synthetically, e.g. by recombinant DNA procedures. Moreover, the term includes any such polypeptide whether its

-15-

sequence is substantially the same as, or identical to the sequence of any mammalian homolog of the human polypeptide, e.g. murine, bovine, porcine, etc. homologs. Additionally, the term includes mutants or other variants of any of the foregoing which retain at least some of the enzymatic activity of nonmutants or nonvariants.

The invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from Axin, but which do not produce phenotypic changes.

However, a mutant Axin will not exhibit the same phenotype as the wildtype Axin. For example, a cell containing a mutant version of the axin gene will express a protein unable to negatively regulate the Wnt signalling pathway.

The nucleic acid of the subject invention also include nucleic acids that encode for polypeptide analogs, fragments or derivatives which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (including deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of the naturally-occurring forms.

The polypeptide of the subject invention also includes analogs, fragments or derivatives which differ from naturally-occurring forms, but Axin activity.

This invention also provides a vector comprising an isolated nucleic acid encoding Axin. The isolated nucleic acid of the vectors is operatively linked to a promoter of RNA transcription which maybe, or is identical to, a bacterial, yeast, insect or mammalian promoter. The vector may be a plasmid, cosmid, yeast artificial chromosome (YAC),

-16-

bacteriophage or eukaryotic viral DNA. Specifically, this invention provides two cosmids designated Genbank Accession No. Z69667 and Z81450, respectively.

5 Further other numerous vector backbones known in the art as useful for expressing proteins may be employed. Such vectors include but are not limited to: adenovirus, simian virus 40 (SV40), cytomegalovirus (CMV), mouse mammary tumor virus (MMTV), Moloney murine leukemia virus, murine sarcoma virus, and Rous sarcoma virus, DNA delivery systems, i.e.
10 liposomes, and expression plasmid delivery systems.

This invention also provides a vector system for the production of a polypeptide which comprises the vector in a
15 suitable host. Suitable host includes a cell which includes, but is not limited, prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells.

20 Suitable animal cells include, but are not limited to, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, Ltk⁻ cells, etc. Expression plasmids such
25 as that described supra may be used to transfect mammalian cells by methods well known in the art such as calcium phosphate precipitation, electroporation.

This invention also provides a method for producing a
30 polypeptide (e.g. Axin) which comprises growing a host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. Methods of recovering polypeptides produced in such host vector systems are well-known in the art and
35 typically include steps involving cell lysis, solubilization and chromatography.

This invention also provides a method of obtaining a

-17-

polypeptide in purified form which comprises: (a) introducing a vector, as described above, into a suitable host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c) recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered. As discussed above the vector may include a plasmid, cosmid, yeast artificial chromosome, bacteriophage or eukaryotic viral DNA. Also, the host cell may be a bacterial cell (including gram positive cells), yeast cell, fungal cell, insect cell or animal cell. Suitable animals cells include, but are not limited to HeLa cells, Cos Cells, CV1 cells and various primary mammalian cells. Culturing methods useful for permitting transformed or transfected host cells to produce polypeptides are well known in the art as are the methods for recovering polypeptides from such cells and for purifying them.

Using the aforementioned method, this invention also provides a purified wildtype Axin and a purified mutant Axin. Further, this invention also provides a polypeptide comprising the amino acid sequence of Axin, including, but limited to, fusion proteins having part of their amino acid sequence the amino acid sequence of Axin.

This invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within a nucleic acid which encodes a wildtype Axin without hybridizing to a nucleic acid which encodes a mutant Axin. Further, this invention also provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin. These oligonucleotides may be DNA or RNA. Such oligonucleotides may be used in accordance with well known standard methods for known purposes, for example, to detect the presence in a sample of DNA which will hybridize thereto.

-18-

As used herein, "capable of specifically hybridizing" means wherein the oligonucleotide will selectively bind to only sequences which are unique to either nucleic acids encoding wildtype or mutant Axin.

5

The oligonucleotides include, but are not limited to, oligonucleotides that hybridize to mRNA encoding Axin so as to prevent translation of the protein.

10 This invention also provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid which encodes Axin. Furthermore, this application also provides an antisense molecule of capable of specifically hybridizing with the isolated nucleic acid
15 encoding mutant Axin.

This invention provides a method for determining whether a subject carries a mutation in the axin gene which comprises
20 (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject carries a mutation in the axin gene.

25 In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic
30 acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from,
35 a nucleic acid which encodes mutant Axin.

As used herein "corresponding to mutant Axin" means capable of specifically hybridizing with a unique sequence of

-19-

nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin.

5 In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated
10 nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine
15 whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step
20 (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

25 One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified
30 nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The
35 detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

-20-

The invention described above may have sample which is blood, tissues or sera.

5 This invention also provides a method for determining whether a subject has a predisposition for cancer which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine
10 whether a subject has a predisposition for cancer.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the
15 determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and
20 (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

As used herein "corresponding to mutant Axin" means capable
25 of specifically hybridizing with a unique sequence of nucleotides present within the nucleic acid which encodes a mutant Axin without hybridizing to a nucleic acid which encodes a wildtype Axin.

30 In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated
35 nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived

-21-

from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

5 In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample
10 is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid
15 present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).
20

25 This invention also provides where the sample includes, but is not limited to, blood, tissues or sera.

As used herein "cancer" includes, but is not limited to, brain, thyroid, breast, colorectal, gastrointestinal, esophageal carcinomas or melanomas.
30

This invention also provides a method for treating a subject who has a predisposition to cancer which comprises
35 introducing the isolated nucleic acid encoding wildtype Axin into the subject under conditions such that the nucleic acid expresses wildtype Axin, so as to thereby treat the subject.

-22-

This invention also provides a method for treating a subject who has a predisposition to cancer which comprises an effective amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby
5 treat the subject who is susceptible to cancer.

This invention also provides a method for determining whether a subject has cancer, which comprises (a) obtaining an appropriate nucleic acid sample from the subject; and (b)
10 determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

15 In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic
20 acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from,
25 a nucleic acid which encodes mutant Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with
30 restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the
35 nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

-23-

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

This invention also provides a method for detecting a mutation in cancerous cells of the subject which comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to

-24-

thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

5 In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of
10 nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a
15 nucleic acid which encodes mutant Axin.

In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step
20 (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

25 One can also practice the invention, wherein the determining of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

30 In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor
35 or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

This invention also provides a method of suppressing cells

-25-

unable to regulate themselves which comprises introducing the isolated nucleic acid encoding wildtype Axin into the cells.

5 In a specific example of the above-described method, the nucleic acid sample in step (a) is mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises (i) contacting the mRNA with the oligonucleotide capable of detecting only nucleic
10 acid corresponding to mutant Axin under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from,
15 a nucleic acid which encodes mutant Axin.

In another specific embodiment, the determining of step (b) comprises (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid encoding Axin with
20 restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid, (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the
25 nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

30 In another embodiment, the determining of step (b) comprises: (i) sequencing the nucleic acid sample of step (a); and (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid encoding wildtype Axin, so as to thereby determine whether the nucleic acid sample
35 is, or is derived from, a nucleic acid which encodes mutant Axin.

One can also practice the invention, wherein the determining

-26-

of step (b) comprises: (i) amplifying the nucleic acid present in the sample of step (a); and (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.

5

In order to facilitate identification of the nucleic acid from step (a) the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker. The detectable marker may be a radioactive isotope, a fluorophor
10 or an enzyme. In additions, the nucleic acid sample may be bound to a solid matrix before performing step (i).

Also, the cells may be unable to regulate themselves because of a mutation in the axin gene or a mutation in the adenomatous polyposis coli gene.
15

This invention also provides a method of suppressing cells unable to regulate themselves which comprises introducing wildtype Axin into the cells in an amount effective enough
20 to suppress the cells.

This invention also provides a method of treating a subject who has cancer which comprises introducing the isolated nucleic acid encoding wildtype Axin, into the subject so as
25 to thereby treat the cancer.

One well-known means of introducing the isolated nucleic acid, comprises: (a) recovering cancerous cells from the subject; (b) introducing the isolated nucleic acid encoding
30 the wildtype Axin into the cells; and (c) reintroducing the cells of step (b) into the subject so as to treat the subject who has cancer.

This invention further provides a method for identifying a
35 chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises: (a) contacting mutant Axin with the chemical compound under conditions permitting binding between the

-27-

mutant Axin and the chemical compound; (b) detecting specific binding of the chemical compound to the mutant Axin; and (c) determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.

This method is applicable when the cells are cancerous. For example, when cancerous cells are derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thyroid or intestine..

This invention provides pharmaceutical compositions comprising the chemical compound identified by the above-described method of in an amount effective to inhibit cancer and a pharmaceutically effective carrier.

Further this invention also includes a pharmaceutical compositions includes an antisense molecule to an isolated nucleic acid which encodes mutant Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

This invention also includes a pharmaceutical composition comprising the purified wildtype Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.

One can treat a subject who has cancer by administering an effective amount of the above-described pharmaceutical composition to the subject who has cancer.

One can administer the above-described compositions by topical, oral, aerosol, subcutaneous administration, infusion, intralesional, intramuscular, intraperitoneal, intratumoral, intratracheal, intravenous injection, or liposome-mediate delivery.

Methods of administration of pharmaceutical compositions are

-28-

well-known in the art.

This invention also includes transgenic, nonhuman mammal containing the isolated nucleic encoding Axin, specifically,
5 an isolated nucleic acid encoding mutant Axin.

This invention further provides a method for treating a subject who has a predisposition to cancer which comprises introducing the isolated nucleic acid which encodes Axin,
10 wherein the Axin is wildtype Axin, into the subject under conditions such that the nucleic acid expresses wildtype Axin, wherein the nucleic acid is overexpressed, so as to thereby treat the subject.

15 This invention provides a method for treating a subject who has cancer which comprises introducing the isolated nucleic acid which encodes Axin, wherein the Axin is wildtype Axin, into the subject under conditions such that the nucleic acid expresses wildtype Axin, wherein the nucleic acid is
20 overexpressed, so as to thereby treat the subject.

In an embodiment of either of the above-described methods the overexpression of the wildtype Axin may be accomplished by putting the introduced nucleic acid under a strong
25 promoter. Methods of inducing overexpression are well known to one of ordinary skill in the art.

This invention provides a method of inhibiting tumorigenesis in a subject which comprises administering an effective
30 amount of a molecule which induces degradation of β -catenin thereby inhibiting tumorigenesis. This method may also be used to inhibit cellular transformation, i.e. oncogenic transformation of a normal cell to a cancer cell. In a preferred embodiment of the above-described methods the
35 molecule which induces degradation of β -catenin is Axin or a functionally active mutant of Axin. Alternatively, drugs

-29-

or small molecules which act with Axin, i.e. in synergy with Axin to induce degradation of β -catenin may also be used in another embodiment of this method, for example drugs which inhibit enzymes.

5

This invention provides a method of treating a subject with cancer which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces degradation of β -catenin and a pharmaceutically acceptable carrier, thereby treating the subject with cancer. In a preferred embodiment of the above-described method the molecule which induces degradation of β -catenin is Axin or a functionally active mutant of Axin. Drugs or small molecules which act with Axin, i.e. in synergy with Axin to induce degradation of β -catenin may also be used in other embodiments of this method, for example drugs which inhibit enzymes.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

25

-30-

FIRST SECTION

Experimental Procedures

5 1. Isolation and characterization of mouse Axin cDNA and genomic clones

Primers for mAxin are named for the position of their 5' terminus in the cDNA (+ numbers) or in upstream genomic DNA (- numbers). F indicates a forward and R a reverse primer. Sequences are listed 5' to 3'. A 315 bp NotI-StuI genomic probe at the left of the transgene insert (Perry et al., 10 1995) was used to screen an E8.5 mouse embryo cDNA library, yielding one clone (N7) that was partially colinear with the probe. Using a fragment of N7, 12 more clones were isolated from a WEHI-3 cDNA library (Stratagene). Additional Axin 15 cDNA clones were obtained from various libraries using probes from clone N7, but none extended as far 5' as N7. 5' RACE was performed using kidney cDNA, AP1 primer (Advantage cDNA PCR kit, Clontech) and mAxin primer +98R (caccagccctctctggaacc). The RACE product extending farthest 20 5' was colinear with clone N7 and contained 4 more bp at the 5' end. To estimate the ratio of forms 1 and 2 mRNA, total RNA was reverse transcribed using oligo-dT primer, and the cDNA was amplified using +2289F (gagggagagaaggagatcag) (Sequence I.D. No. 5) and +2744R 25 (gtagctccccttcttggttag) (Sequence. I.D. No. 6).

Intron/exon structure was determined by restriction mapping and sequencing of genomic subclones and products of long template PCR using primers in adjacent exons. Previously 30 isolated clones (Perry et al., 1995) included exons 1-3, and clones including exons 6-10 were isolated from a strain 129 library. The remaining region was isolated by long template PCR (Boehringer Mannheim) using primers at different positions in the cDNA.

35

2. Isolation of human and chick Axin cDNA clones

Database searches revealed ESTs (T07178, R75687, T30966, T32063, T15895 and T72547) representing the 3' region of a

-31-

human Axin homolog (*hAxin*). Additional clones were isolated by 5' and 3' RACE using human placenta RNA (Clontech Marathon RACE kit). A stage 12-15 chick embryo cDNA library (a gift of Dr. D. Wilkinson) was screened with a *mAxin* probe and four clones containing the same insert in both orientations were isolated. The 3131 bp *cAxin* cDNA sequence contains a polyadenylation signal near the 3' end, but is shorter than the *cAxin* mRNA (~3.6 kb), even after accounting for a poly(A) tail, and thus may lack part of the 5' UTR.

3. Sequence analysis

Database searches were conducted using BLAST, and sequence alignments using ClustalW and BOXSHADE. Other RGS sequences are: Q08116 (*hRGS1*), P41220 (*hRGS2*), U27768 (*hRGS3*), U27768 (*rRGS4*), D31257 and R35272 (*hRGS5*), U32328 (*hRGS7*), H87415 (*hRGS10*), X91809 (*hGAIP*).

4. Northern blot and *in situ* hybridization

20 µg of total RNA from embryos, adult tissues or ES cells was run on a formaldehyde-agarose (1.2%) gel, blotted to Genescreen plus (NEN-Dupont), and hybridized with a ³²P-DNA probe containing the entire *mAxin* cDNA sequence, as described (Perry et al., 1995). For *in situ* hybridization (Wilkinson, 1992), an anti-sense probe was produced by T7 transcription of a HindIII-SacI fragment of *mAxin* cDNA (bp 765-1065, within exon 2) in pBluescript. A sense probe did not produce a significant signal. The *HNF-3β* probe was produced from clone c21 (Sasaki and Hogan, 1993).

5. Constructs for *Xenopus* injection

Axin cDNAs were cloned into the XhoI site of pCS2+MT (Rupp et al., 1994). The experiments shown employed vector MTPA2, which includes *Axin* form 2 (bp 37 to 3310) and encodes aa 13 to the normal C-terminus. Translation initiates in the N-terminal Myc tag. Three other *Axin* vectors (MTPA1, MTFU1 and MTFU2) were similarly active at ventralization: MTPA1 was identical to MTPA2 except it was derived from a form 1 cDNA. MTFU1 and MTFU2 were identical to MTPA1 and MTPA2, except

-32-

they contained a longer 3' UTR (bp 3311-3731). Δ RGs was derived from MTFU1 by deleting cDNA bp 754 -1053. *Siamois* was cloned in pCS2+MT (Fagotto et al., 1997) and β -catenin (C-terminally HA-tagged) in pSP36 (Funayama et al., 1995).
5 Other expression vectors were: *Xwnt8* and *Noggin* (gift of Dr. R. Harland), *Activin* (Dr. D. Melton), Δ BMPR (Dr. A. Suzuki), *dnGSK-3* (GSK-3-K->R, Dr. D. Kimelman) and *Xdsh* (Dr. U. Rothbacher). GSK-3-K->R is a mutant of *Xenopus* GSK-3 β without kinase activity (Pierce and Kimelman, 1995). Δ BMPR
10 is a truncated BMPR lacking the kinase domain (Suzuki et al., 1994).

6. *Xenopus* injections and analysis of phenotypes
mRNAs were synthesized and injected as previously described
15 (Fagotto et al., 1996; 1997). For RT-PCR, mRNA was extracted from whole early gastrulae (stage 10 1/2) or dissected dorsal and ventral halves, and specific mRNAs were detected as described (Fagotto et al., 1997). *Siamois* primers were:
20 5' ttgggagacagacatga (corresponds to part of the 5' UTR, present in the endogenous mRNA but not the injected synthetic *Siamois* mRNA) (Sequence I.D. No. 7) and 3':
tcctgttgactgcagact (Sequence I.D. No. 8). Other primers were as described (Fagotto et al., 1997). For immunofluorescence,
25 Myc-tagged Axin was detected in frozen sections of early gastrulae using anti-Myc antibody 9E10.2, as described (Fagotto et al., 1996; Fagotto and Gumbiner, 1994).

Results

30 1. Identification of the Axin gene

A genomic probe from the *Axin*^{Tg1} transgene insertion locus detected a 3.9 kb RNA in wild type embryonic stem (ES) cells but not in *Axin*^{Tg1/Tg1} ES cells, representing a strong candidate for the Axin mRNA (Perry et al., 1995). To isolate
35 cDNA clones, a probe located within a CpG island upstream from the transgene insertion site (Fig. 1) was used to screen a mouse embryo library. One cDNA clone contained a region identical in sequence with the genomic probe,

-33-

confirming that it was encoded at the Axin locus, and this clone was used to isolate additional overlapping cDNA clones. The composite cDNA sequence was 3623 bp long, excluding the poly(A) tail. All the cDNA clones were co-linear in their regions of overlap, except for a 108 bp sequence present in a fraction of clones following bp 2579, representing an alternative splicing product (Fig. 1). Transcripts lacking this 108 bp segment were termed "form 1" and those containing it "form 2".

2. Genomic organization of Axin

Using Axin cDNA probes, a series of overlapping clones was isolated from a WT mouse genomic library, and the locations of exons and introns were determined (see Experimental Procedures). As illustrated in Fig. 1, 10 exons were identified, spanning ~56 Kb. The extra 108 bp segment in form 2 mRNA results from the use of an alternative 5' splice site following exon 8, and is designated exon 8A. The longest cDNA clones representing the 3.9 kb mRNA appeared to be missing 25-75 nt from the 5' end, based on RNase H and S1 nuclease mapping studies. Difficulties in cloning these 5' terminal sequences may be a consequence of the very high GC content of the CpG island surrounding the apparent promoter region (Fig. 1). Based on Southern blot hybridization to genomic DNA, and the analysis of multiple cDNA and genomic clones, Axin appears to be a single copy gene.

In the *Axin*^{Tg1} allele, exon 2 and parts of the two flanking introns are deleted. Exons 1 and 3 are separated by a ~600 kb transgene insertion (Fig. 1), a disruption that leads to the absence of the major WT 3.9 kb mRNA in homozygotes (Fig. 2). As described elsewhere (Vasicek et al., manuscript submitted), the *Axin*^{Pu} allele contains an endogenous intracisternal A particle (IAP) provirus within intron 6, while *Axin*^{Rb} contains a similar IAP element interrupting exon 7. The *Axin*^{Ki} allele is apparently extinct.

3. Ubiquitous expression of wild type Axin mRNA.

-34-

On Northern blots, a major band of ~3.9 kb was observed in all WT adult tissues examined, embryos at E10.5 - E16.5, and ES cells. A 3.0 kb band was also observed at very low levels in some WT tissues and ES cells. In *Axin^{Tg1/Tg1}* ES cells, the 3.9 kb RNA was absent, but a 3.0 kb RNA was observed (Fig. 2A). Because the 3.0 kb mRNA was observed in both WT and *Axin^{Tg1/Tg1}* cells, and contains exons 3 - 10 but not 1 and 2, it is likely to be transcribed from a weak promoter downstream from the 3' end of the transgene-induced deletion. Thus, *Axin^{Tg1}* is a loss-of-function allele with respect to the major 3.9 kb mRNA, although it may not be a null allele.

In situ hybridization with WT embryos at E7.5 - E9.5 showed that *Axin* mRNA is uniformly distributed throughout embryonic and extraembryonic tissues of the postimplantation embryo (Fig. 2B). *Axin* mRNA was also detected by RT-PCR in 1-cell through blastocyst stage embryos. Form 1 and 2 mRNAs were both present in all adult tissues examined and in ES cells (Fig. 2C).

20

4. The predicted amino acid sequences of Axin and its human and chicken homologs

The murine *Axin* (*mAxin*) cDNA sequence included an open reading frame (ORF) beginning at base 3, which could encode a protein of up to 956 (form 1) or 992 (form 2) amino acids (Fig. 3A). Homology searches identified several ESTs representing a human *Axin* homolog, and additional human cDNA sequences were isolated by 5' and 3' RACE (Chenchik et al., 1995). The predicted human and murine *Axin* amino acid (aa) sequences are 87% identical overall. In addition, a cDNA clone representing a chicken homolog was isolated, and its predicted aa sequence was 66% identical to mouse *Axin* (Fig. 3A). The first two AUG codons in the *mAxin* ORF were located at bp 375 and 391 of the cDNA, but neither was surrounded by a consensus initiation site (Kozak, 1986). To determine whether either site could serve as an initiation codon, 293T cells were transfected with *Axin* cDNA expression vectors including a C-terminal epitope tag. The sizes of the *in vivo*

-35-

translation products were consistent with initiation at one of the first two AUG codons. However, because the murine and human ORFs continue to be conserved upstream of this position, it remains possible that the normal initiation site is further upstream.

The predicted aa sequence contains multiple sites for Ser/Thr phosphorylation and one for Tyr phosphorylation, suggesting that Axin may be a phosphoprotein. It also contains one (form 2) or two (form 1) sequences matching the consensus for a bipartite nuclear localization signal (NLS) (Dingwall and Laskey, 1991). However, detection of epitope-tagged Axin proteins expressed in mammalian cells or *Xenopus* embryos indicated a peri-membrane rather than a nuclear location for both forms. Database searches revealed two regions of homology to other known proteins. One of these, aa 213 to 338, shows 30-40% identity and 50-60% similarity to the RGS (Regulation of G-protein Signaling) domain (Dohlman and Thorner, 1997). A second potentially important region of similarity (Fig 3c) is a 51 aa segment near the Axin C-terminus, which is ~40% identical and ~60% similar to a conserved sequence near the N-terminus of *Drosophila* Dsh and its vertebrate homologs (Klingensmith et al., 1994; Sussman et al., 1994). Both the RGS and Dsh homology regions are highly conserved among mouse, human and chick Axin homologs (Fig. 3A).

5. Injection of Axin mRNA inhibits dorsal axis formation in *Xenopus* embryos

The observation that the *Axin*^{Tg1} and *Axin*^{Kb} alleles, which cause axial duplications in homozygous mouse embryos, are both unable to produce the major 3.9 kb mRNA suggested that one function of Axin is to negatively regulate an early step in axis formation. Because the Axin sequence is highly conserved among amniotes, mAxin might be able to function in amphibian embryos, a system highly amenable to experimental manipulation of early axial development. Therefore, *in vitro* synthesized mAxin mRNA (encoding aa 13-956, with an N-

-36-

terminal Myc epitope tag) was injected into the dorsal, subequatorial region of 4-cell *Xenopus* embryos, which were scored at the tadpole stage for effects on axis formation (Fig. 4A and Table 1). Most of these embryos developed with strong axial defects ranging from loss of anterior structures to complete lack of body axis, a phenotype characteristic of completely ventralized embryos (Kao and Elinson, 1988). Control injections of β -gal mRNA had no effect. Embryos injected dorsally with Axin also showed a markedly reduced expression of the dorsal markers *Siamois*, *Goosecoid*, *Chordin* and *Xnr3*, consistent with the observed ventralizing effects (Fig. 4B). Forms 1 and 2 Axin mRNAs were equally active, and α -Myc staining showed that both proteins were similarly localized in a punctate pattern near the plasma membrane.

Table 1. Frequency and extent of ventralization by dorsal injection of Axin mRNA, and rescue by β -catenin or *Siamois* but not *Xwnt8*.

Both dorsal blastomeres of 4-cell embryos were injected in the subequatorial region. Dorso-Anterior Index (DAI) is a measure of axial development; where 5 is normal, 0 is completely ventralized, and >5 is hyper-dorsalized (Kao and Elinson, 1988). * DAI < 4 was considered significant ventralization.

mRNAs injected	%	Average DAI	Number
β -galactosidase (2 ng, control)	3	4.8	31
Axin (2 ng)	78	1.7	118
Axin + <i>Xwnt8</i> (10-20 pg)	90	1.9	67
Axin + β -catenin (300 pg)	29	4.3	35
Axin + <i>Siamois</i> (50-100 pg)	2	5.1	96

6. Inhibition of dorsal axis formation by Axin is mediated by the Wnt signaling pathway

The ventralizing effect of Axin could be due either to

-37-

inhibition of Nieuwkoop Center activity, which requires the activation of the Wnt signaling pathway, or to perturbation of further downstream inductive processes, i.e. establishment of the Spemann organizer or BMP-dependent mesodermal patterning. To test whether Axin might exert its effects via the Wnt pathway, mRNA encoding *Xwnt8*, β -catenin or *Siamois* was co-injected with Axin mRNA into the dorsal, subequatorial region. *Siamois* is a homeobox gene whose expression is specifically activated by Wnt signaling, and which appears to mediate the effects of the Wnt pathway on axis formation (Carnac et al., 1996; Fagotto et al., 1997; Lemaire et al., 1995). Co-injection of *Siamois* or β -catenin, but not *Xwnt8*, overcame the ventralizing effect of Axin, rescuing normal axis formation in a large proportion of embryos (Fig. 4A and Table 1) and restoring expression of dorsal markers (Fig. 4C).

As *Xwnt8* or several downstream factors can induce a secondary dorsal axis when injected into the ventral side of the embryo, the ability of Axin to inhibit secondary axis formation was examined. Co-expression of Axin completely inhibited the axis-inducing activity of *Xwnt8*, *Xdsh* (a *Xenopus* Dsh homolog) and dominant-negative GSK-3, while it did not affect secondary axis formation by β -catenin or *Siamois* (Figs. 5A, and B). Thus, injection of Axin mRNA can block either normal or secondary dorsal axis formation in *Xenopus* embryos, apparently by interfering with signaling through the Wnt pathway at a level downstream of Wnt, Dsh and GSK-3, and upstream of β -catenin and *Siamois*.

7. Expression of Axin does not affect other downstream pathways involved in axis formation.

Induction of the Spemann organizer can be also mimicked by Activin, a potent mesodermal inducer, which at high concentrations induces dorsal mesoderm. Axin did not inhibit the induction of *Goosecoid* by Activin in the ventral region of early gastrula embryos (Fig. 5C), and had no effect on the formation of an ectopic blastopore lip or a partial

secondary axis in Activin-injected embryos (Fig. 5A). These results are consistent with the conclusion that Activin acts downstream of, or in parallel to, the Wnt pathway (Carnac et al., 1996; Fagotto et al., 1997; Wylie et al., 1996).

5

Axial patterning is also regulated further downstream by the antagonistic activity of factors secreted by the organizer (Noggin, Chordin, Follistatin) and the ventral mesoderm (BMPs). For instance, ventral expression of Noggin, a natural inhibitor of BMPs, or a dominant-negative truncated BMP receptor (Δ BMPR) causes formation of an ectopic axis (Hogan, 1996). However, Axin failed to block the induction of a secondary axis, or the ectopic expression of the dorsal marker *Goosecoid*, by ventral injection of *Noggin* or Δ BMPR (Figs. 5A, C). These results confirm that Axin acts specifically on the Wnt signaling pathway, and does not perturb other pathways involved in early axial patterning.

8. Deletion of the *Axin* RGS homology region creates a dominant-negative mutant.

To test the importance of the RGS domain, we injected *Xenopus* embryos with mRNA encoding Δ RGS, a mutant form of Axin in which the sequences encoding aa 252-351 were deleted. Dorsal injection of Δ RGS revealed that it had lost the ability to ventralize (only 4/44 embryos ventralized, average Dorso-Anterior Index ~ 5). Surprisingly, Δ RGS acted as a potent dorsalizer when injected ventrally, producing secondary axes (usually complete, including the most anterior head structures) in 87% of embryos (Figs. 6A-C). Δ RGS induced ectopic expression of several dorsal markers, including *Siamois*, consistent with an activation of the Nieuwkoop Center signaling pathway (Fig. 6D). Ventral injection of wild type Axin mRNA had no effect on development and did not induce ectopic expression of dorsal markers (Figs. 6C, and D). However, co-injection of Axin blocked the axis-inducing activity of Δ RGS, as did co-injection of *C-cadherin*, which binds to and inhibits signaling through β -catenin (Fagotto et al., 1996) (Fig.

-39-

6C). Thus, Δ RGs has an effect opposite to that of Axin, and appears to act through a dominant-negative mechanism to inhibit an endogenous Axin activity. The axial duplications induced by Δ RGs are reminiscent of those caused by loss-of-function Axin alleles in the mouse embryo, two examples of which are shown in Figs. 6 (E-G).

Discussion

Mutations at the *Fused* locus have been a subject of interest since the early days of mouse genetics because of their pleiotropic effects on a variety of developmental processes. The most remarkable abnormality seen in early post-implantation embryos homozygous for *Fused* alleles was the formation of ectopic axial structures, which led to the suggestion that this locus played a role in the determination of the embryonic axis (Gluecksohn-Schoenheimer, 1949). The wild type *Fused* gene, now called Axin, and a transgenic insertional mutant allele, have been cloned and the structure characterized of Axin^{Tg1}. Two of the old mutant alleles, Axin^{Fu} and Axin^{fb}, have also been characterized (Vasicek et al., submitted). The observation that the major Axin mRNA is disrupted in two different alleles that cause axial duplications suggested that the normal gene product plays a negative regulatory role at some step in axis formation. This hypothesis is supported by the observation that dorsal injection of Axin mRNA blocks axis formation in *Xenopus* embryos, while ventral injection of a dominant-negative form of Axin induces a complete secondary axis. Furthermore, co-injection of Axin with factors in the Wnt signal transduction pathway shows that Axin exerts its effects on axis formation by inhibiting the Wnt pathway. These studies reveal that Axin is a novel regulatory protein for a signaling pathway known to trigger an early step in embryonic axis formation in amphibians. Results, together with the phenotype of Axin mutant embryos, also imply that the Wnt signaling pathway plays an early and critical role in axis formation in mammalian embryos.

-40-

1. The *Axin* gene

The *Axin* gene encodes a major mRNA of 3.9 kb, which is expressed ubiquitously in embryos and adult tissues. In the *Axin*^{Tg1} allele, exon 2 is replaced with ~600 kb of transgene DNA, preventing expression of the major mRNA. Two spontaneous *Axin* alleles, *Axin*^{Fv} and *Axin*^{Kb}, are each caused by the insertion of an IAP provirus, within intron 6 or exon 7, respectively (Vasicek et al., submitted). Many of the similarities and differences between the phenotypic effects of *Axin* alleles can be explained by the nature of these mutations. While the provirus in the *Axin*^{Fv} intron is efficiently spliced out, resulting in near-normal levels of the 3.9 kb mRNA, the provirus in *Axin*^{Kb} precludes the production of the normal mRNA. Therefore, the similar recessive defects and embryonic lethality seen in *Axin*^{Tg1} and *Axin*^{Kb} (but not *Axin*^{Fv}) embryos can be attributed to the inability of either allele to encode the major *Axin* mRNA. On the other hand, the dominant effects of *Axin*^{Fv} and *Axin*^{Kb}, which are not seen in *Axin*^{Tg1} mice, appear to be a specific consequence of abnormal transcripts associated with the proviral insertions in these alleles (Vasicek et al., submitted).

Two genomic cosmid clones encoding part of a human *Axin* homolog map to chromosome 16p13.3 (Accession No. Z69667 and Z81450). Examination of the human genetic map did not reveal any genetic traits (e.g., developmental or neurological defects) that seem likely to be associated with *Axin* mutations.

The predicted *Axin* protein includes regions of similarity to two families of proteins involved in signal transduction, RGS and Dsh. Several proteins containing an RGS domain (De Vries et al., 1995; Druey et al., 1996; Koelle and Horvitz, 1996) bind G_α subunits (De Vries et al., 1995; Dohlman et al., 1996) and serve as GTPase-activating proteins (GAPs) for the G_i subfamily of G_α subunits, thus inhibiting signal transduction by accelerating the rate of the intrinsic GTPase (Berman et al., 1996; Hunt et al., 1996; Watson et

-41-

al., 1996). At least 17 mammalian RGS proteins have been identified, and it is not yet clear if they all serve as GAPs for members of the $G_{i/o}$ subfamily, or if some serve as GAPs for other G_o subfamilies, or perform other functions (Dohlman and Thorner, 1997). While the Axin RGS domain contains similar residues at many positions of amino acid conservation among RGS proteins, it differs at other conserved positions, and contains two short inserts not present in other RGS domains (Fig. 3B). Thus, whether the Axin RGS is a G_o GAP remains to be determined. Axin also displays homology to a 50 aa sequence within a conserved N-terminal region of Drosophila and vertebrate Dsh proteins. The importance of this sequence is unknown, although deletion of a 165 aa segment including this sequence rendered the Drosophila protein inactive (Yanagawa et al., 1995).

2. Axin and Wnt signaling in establishment of the amphibian Nieuwkoop Center

Based on its ability to block ectopic axis formation in *Xenopus* embryos by Xwnt8, Dsh or dnGSK-3, Axin appears to negatively regulate signaling through the Wnt pathway, either at the level of GSK-3 or further downstream. Furthermore, its inability to block the effects of β -catenin or Siamois suggests that Axin acts upstream of β -catenin (Fig. 7). GSK-3 is a Ser/Thr protein kinase whose activity results in the phosphorylation of β -catenin and its consequent degradation. GSK-3 may directly phosphorylate β -catenin (Yost et al., 1996), or its effects on β -catenin may be mediated by the phosphorylation of adenomatous polyposis coli (APC), which associates with β -catenin and GSK-3 (Miller and Moon, 1996). When GSK-3 activity is inhibited, either naturally through the activity of Wnt and Dsh, or experimentally by dnGSK-3, the level of cytosolic β -catenin is increased, downstream effectors are activated, and Nieuwkoop Center activity results. Thus, both Axin and GSK-3 negatively regulate formation of the Nieuwkoop Center by inhibiting the same signaling pathway.

-42-

One hypothetical mechanism for the similar action of Axin and GSK-3 (Fig. 7) is suggested by the observation that Axin can bind to the Ser/Thr protein phosphatase PP2A. If Axin were to inhibit PP2A activity, and if PP2A de-phosphorylated the GSK-3 substrate(s) involved in Wnt signaling, then the overexpression of Axin would increase the level of phosphorylation of this substrate. Thus, even if GSK-3 activity were reduced by Dsh or dnGSK-3, the substrate would remain highly phosphorylated, which could explain why Axin appears epistatic to these two proteins. Alternatively, Axin might stimulate GSK-3 activity by another mechanism that can overcome its inhibition by Dsh or dnGSK-3. A third possibility is suggested by the demonstrated importance of the RGS domain for the ability of Axin to inhibit Wnt signaling and ventralize the frog embryo. There is no evidence for a G_q protein in the Wnt pathway, and the evidence that Axin functions downstream of Dsh and GSK-3 would argue against an activity at the level of a hypothetical G-protein coupled to a Wnt receptor. However, the Axin RGS domain, if it is a functional G_q GAP, might inhibit a second signaling pathway involving a G_q protein, which converges with and stimulates the Wnt pathway at a level downstream of GSK-3. In order to explain the ability of high levels of Axin to block the Wnt pathway, this second signal would have to be required for some step in the transmission of the Wnt signal (e.g., cytosolic accumulation of β -catenin) in the early frog embryo.

Not only does deletion of the RGS region abolish the axis-inhibiting properties of Axin, but it creates a dominant-negative form that can induce an ectopic dorsal axis. An obvious implication is that the amphibian embryo contains a protein homologous to Axin, which normally serves to inhibit ectopic axis formation, and whose activity is blocked by Δ RGS. This conclusion is consistent with the observation that loss-of-function Axin mutations in the mouse cause the development of ectopic axial structures. Thus, not only is Axin capable of inhibiting signaling through the Wnt pathway

-43-

when it is over-expressed, but this appears to be a natural function of the protein. Presumably, the levels of endogenous Axin in the embryo are high enough to prevent signaling in the absence of a strong upstream signal (e.g.,
5 a Wnt ligand), but low enough to allow signaling when the pathway is activated by a natural or experimental stimulus. While the dominant-negative mechanism of Δ RGS remains to be determined, one possibility is that it competes for binding to a protein to which Axin normally binds, but fails to
10 perform the function carried out by the RGS domain.

3. Axin, Wnts and axis formation in mammalian embryos

The ability of Axin to regulate an early step in *Xenopus* axis formation mediated by the Wnt signaling pathway,
15 together with the occurrence of axial duplications in Axin mutant mouse embryos, argues strongly that an evolutionarily conserved mechanism involving the Wnt pathway plays a critical role in embryonic axis formation in mammals as well as amphibians. In *Xenopus*, signaling through components of
20 the Wnt pathway is thought to confer Nieuwkoop Center activity to a group of dorsal-vegetal blastomeres, which consequently secrete factors that induce formation of the organizer by the adjacent dorsal mesoderm. In the mouse, the equivalent of the organizer is the node, a group of cells at
25 the anterior end of the primitive streak (Beddington, 1994). Fate mapping of the pre-streak embryo indicates that the node derives from epiblast cells at the future posterior pole (Lawson et al., 1991). While the location of the Nieuwkoop Center equivalent (i.e., the cells that induce
30 formation of the organizer) is unknown, the most likely locations are the posterior extraembryonic or embryonic ectoderm proximal to the cells fated to form the node, or the overlying visceral endoderm (Bachvarova, 1996; Conlon and Beddington, 1995).

35

During normal mouse embryogenesis, the Wnt signaling pathway is activated in a discrete region of the early egg cylinder, by the localized production of a Wnt or another stimulus

-44-

that activates downstream components of the Wnt pathway. This localized signal establishes the A-P axis of the embryo, and the responding cells constitute a Nieuwkoop Center equivalent (NCE). According to this model, the

5 ubiquitously expressed Axin serves to attenuate the response to this signal, so that cells in regions of the embryo not exposed to the signal, or exposed to low levels, do not respond, and only a single NCE is formed at the appropriate developmental stage. In mutant embryos lacking Axin, the Wnt

10 pathway could be inappropriately activated and multiple NCE would result. The presence of multiple, discrete axes in mutant embryos could be explained by lateral inhibition mechanism, whereby once a NCE or organizer is formed, it restricts axis formation in adjacent regions of the embryo

15 (Cooke, 1972; Khaner and Eyal-Giladi, 1989; Ziv et al., 1992).

The hypothesis that the Wnt pathway is important for mammalian axis formation is supported by the observation

20 that ubiquitous expression of *Cwnt-8C* in transgenic embryos causes axial duplications (Pöpperl et al., 1997). Also consistent is the failure of β -catenin null mutant mouse embryos to undergo gastrulation (Haegel et al., 1995). No

25 *Wnt* mutants produced so far have affected early events in axis formation, and the expression of *Wnts* in pre-streak mouse embryos has not been reported (Moon et al., 1997; Parr and McMahon, 1994), leaving open the question of what signal triggers this pathway in the normal embryo. In the pre-

30 streak chick embryo, on the other hand, *Cwnt-8c* is expressed in the posterior marginal zone, a region possibly equivalent to the Nieuwkoop Center (Hume and Dodd, 1993).

While the earliest stage at which axial duplications originate in Axin mutant embryos has not been defined, they

35 appear to occur by the stage at which NCE activity would be expected, i.e., the early egg cylinder. Some of earliest *Axin*^{K1/K1} embryos examined (E7.0 - E7.5) contained a bifurcated epiblast with two discrete amniotic cavities or

-45-

two primitive streaks (Gluecksohn-Schoenheimer, 1949; Tilghman, 1996). At later stages, in addition to duplication of anterior axial mesoderm (e.g., Figs. 7F, and G), duplication of the allantois (derived from posterior streak) has been observed in both *Axin^{Tg1}* and *Axin^{Ki}* embryos (Gluecksohn-Schoenheimer, 1949; Perry et al., 1995). In contrast, transplantation of the node to an ectopic site resulted in formation of ectopic notochord, neural tube and somites, but not allantois (Beddington, 1994). Therefore, structures derived from the most anterior and posterior portions of the primitive streak are duplicated in *Axin* mutant embryos, suggesting that the duplications precede the formation of the streak. The extent of anterior neuroectodermal development of the ectopic axes in *Axin* mutant embryos remains to be further studied using molecular markers: "complete twinning" as well as partial duplications were reported in *Axin^{Ki}* embryos, but ectopic forebrain structures have not yet been documented in *Axin^{Tg1}* or *Axin^{Kb}* embryos.

In addition to their effects on axis formation, *Axin* mutations cause neuroectodermal defects (incomplete closure, malformation or truncation of the head folds), cardiac defects and embryonic lethality in homozygotes. It remains to be determined whether these abnormalities are also due to defective regulation of Wnt signaling pathways. Anterior truncations have been observed in transgenic mouse embryos that ubiquitously expressed *Cwnt-8C* (R. Beddington, personal communication) and in frog embryos ectopically expressing *Xwnt-8* after the mid-blastula transition (Christian and Moon, 1993). Therefore, inappropriate Wnt signaling may also account for the neuroectodermal defects in *Axin* mutant embryos. Another interesting question that can now be addressed is the molecular basis of the dominant defects seen in *Axin^{Nu}*, *Axin^{Kb}* and *Axin^{Ki}* heterozygotes, which were attributed to gain-of-function mutations (Greenspan and O'Brien, 1986). Analysis of *Axin^{Nu}* and *Axin^{Kb}* suggests that their similar dominant effects may be mediated by C-

-46-

terminally truncated Axin proteins that are potentially encoded by abnormally spliced transcripts (Vasicek et al., manuscript submitted). It is possible that these abnormal Axin proteins perturb Wnt signaling pathways involved in
5 brain and skeletal development.

REFERENCES FOR THE FIRST SECTION

- Bachvarova, R. F. (1996) "Anterior-posterior polarization
10 and mesoderm inducing factors in the pregastrula mouse embryo." Comparison to chick and frog embryos. Adv. Dev. Biol. 4: 147-191.
- Beddington, R. S. P. (1994) "Induction of a second neural axis by the mouse node." Development 120: 613-620.
- 15 Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) "GAIP and RGS4 are GTPase-activating proteins for the G_i subfamily of G protein α subunits." Cell 86: 445-452.
- Carnac, G., Kodjabachian, L., Gurdon, J. B., and Lemaire, P. (1996) "The homeobox gene Siamois is a target of the Wnt
20 dorsalization pathway and triggers organizer activity in the absence of mesoderm." Development 122: 3055-3065.
- Chenchik, A., Moqadam, J., and Siebert, P. (1995) "Marathon cDNA amplification: a new method for cloning full-length cDNAs." CLONETECHniques 10: 5-8.
- 25 Christian, J. L., and Moon, R. T. (1993) "Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus." Genes Dev 7: 13-28.
- Conlon, F., and Beddington, R. (1995) "Mouse gastrulation
30 from a frog's perspective." Semin. Dev. Biol. 6: 249-256.
- Cooke, J. (1972) "Properties of the primary organization field in the embryo of Xenopus laevis. II. Positional information for axial organization in embryos with two head organizers." J Embryol Exp Morphol 28: 27-46.
- 35 De Vries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995) "GAIP, a protein that specifically interacts with the trimeric G protein G α i3, is a member of a protein family with a highly conserved core domain." Proc

- Natl Acad Sci U S A 92: 11916-11920.
- Dingwall, C., and Laskey, R. A. (1991) "Nuclear targeting sequences--a consensus?" Trends Biochem Sci 16: 478-81.
- Dohlman, H. G., Song, J., Ma, D., Courchesne, W. E., and
5 Thorners, J. (1996) "Sst2, a negative regulator of
pheromone signaling in the yeast *Saccharomyces cerevisiae*:
expression, localization, and genetic interaction and
physical association with Gpa1 (the G-protein α subunit)." Molec. Cell. Biol. 16: 5194-5209.
- 10 Dohlman, H. G., and Thorners, J. (1997) "RSG proteins and
signaling by heterotrimeric G proteins." J. Biol. Chem.
272: 3871-4.
- Druey, K. M., Blumer, K. J., Kang, V. H., and Kehrl, J. H.
(1996) "Inhibition of G-protein-mediated MAP kinase
15 activation by a new mammalian gene family." Nature 379:
742-746.
- Fagotto, F., Funayama, N., Gluck, U., and Gumbiner, B. M.
(1996) "Binding to cadherins antagonizes the signaling
activity of beta-catenin during axis formation in
20 *Xenopus*." J. Cell Biol. 132: 1105-1114.
- Fagotto, F., Guger, K., and Gumbiner, B. M. (1997)
"Induction of the primary dorsalizing center in *Xenopus* by
the Wnt/GSK/ β -catenin signaling pathway, but not by Vg1,
Activin, or Noggin." Development 124: 453-460.
- 25 Fagotto, F., and Gumbiner, B. M. (1994) "Beta-catenin
localization during *Xenopus* embryogenesis: accumulation at
tissue and somite boundaries." Development 120: 3667-79.
- Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B. M.
(1995) "Embryonic axis induction by the armadillo repeat
30 domain of beta-catenin: evidence for intracellular
signaling." J. Cell Biol. 128: 959-68.
- Gardner, R. L., Meredith, M. R., and Altman, D. G. (1992)
"Is the anterior-posterior axis of the fetus specified
before implantation in the mouse?" J. Exp. Zool. 264: 437-
35 443.
- Gluecksohn-Schoenheimer, S. (1949) "The effects of a lethal
mutation responsible for duplications and twinning in
mouse embryos." J. Exp. Zool. 110: 47-76.

- Greenspan, R. J., and O'Brien, M. C. (1986) "Genetic analysis of mutations at the fused locus in the mouse." Proc Natl Acad Sci U S A 83: 4413-7.
- 5 Haegel, H., Larue, L., Ohsugi, M., Fedorov, L., Herrenknecht, K., and Kemler, R. (1995) "Lack of beta-catenin affects mouse development at gastrulation." Development 121: 3529-3537.
- 10 Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C. Y., and Wylie, C. (1994) "Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos." Cell 79: 791-803.
- 15 Hogan, B. L. (1996) "Bone morphogenetic proteins: multifunctional regulators of vertebrate development." Genes Dev 10: 1580-94.
- Hoppler, S., Brown, J. D., and Moon, R. T. (1996) "Expression of a dominant-negative Wnt blocks induction of *MyoD* in *Xenopus* embryos." Genes Dev 10: 2805-17.
- 20 Hume, C. R., and Dodd, J. (1993) "Cwnt-8C: a novel Wnt gene with a potential role in primitive streak formation and hindbrain organization." Development 119: 1147-1160.
- Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) "RGS10 is a selective activator of $G\alpha_i$ GTPase activity" Nature 383: 175-177.
- 25 Jacobs-Cohen, R. J., Spiegelman, M., Cookingham, J. C., and Bennett, D. (1984) "Knobbly, a new dominant mutation in the mouse that affects embryonic ectoderm organization." Genet. Res. 43: 43-50.
- 30 Kao, K. R., and Elinson, R. P. (1988) "The entire mesodermal mantle behaves as Spemann's organizer in dorsoanterior enhanced *Xenopus laevis* embryos." Dev Biol 127: 64-77.
- Khaner, O., and Eyal-Giladi, H. (1989) "The chick's marginal zone and primitive streak formation. I. Coordinative effect of induction and inhibition." Dev Biol 134: 206-214.
- 35 Klingensmith, J., Nusse, R., and Perrimon, N. (1994) "The *Drosophila* segment polarity gene *dishevelled* encodes a novel protein required for response to the wingless

- signal." Genes Dev 8: 118-30.
- Koelle, M. R., and Horvitz, H. R. (1996) "EGL-10 regulates G protein signaling in the *C. elegans* nervous system and shares a conserved domain with many mammalian proteins." Cell 84: 115-125.
- 5 Kozak, M. (1986) "Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes." Cell 44: 283-92.
- Lawson, K. A., Meneses, J. J., and Pedersen, R. A. (1991)
- 10 "Clonal analysis of epiblast fate during germ layer formation in the mouse embryo." Development 113: 891-911.
- Lemaire, P., Garrett, N., and Gurdon, J. B. (1995)
- "Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to
- 15 induce a complete secondary axis." Cell 81: 85-94.
- Lyon, M. F., Rastan, S., and Brown, S. D. M. (1996) Genetic Variants and Strains of the Laboratory Mouse (Oxford: Oxford University Press).
- Miller, J. R., and Moon, R. T. (1996) "Signal transduction through β -catenin and specification of cell fate during
- 20 embryogenesis." Genes Dev. 10: 2527-2539.
- Moon, R. T., Brown, J. D., and Torres, M. (1997) "WNTs modulate cell fate and behavior during vertebrate development." Trends Genetd. 13: 157-162.
- 25 Parr, B. A., and McMahon, A. P. (1994) "Wnt genes and vertebrate development." Curr. Opin. Genet. Dev. 4: 523-8.
- Perry, W. L. I., Vasicek, T. J., Lee, J. J., Rossi, J. M., Zeng, L., Zhang, T., Tilghman, S. M., and Costantini, F. (1995) "Phenotypic and molecular analysis of a transgenic
- 30 insertional allele of the mouse *Fused* locus." Genetics 141: 321-332.
- Pierce, S. B., and Kimelman, D. (1995) "Regulation of Spemann organizer formation by the intracellular kinase *Xgsk-3*." Development 121: 755-765.
- 35 Pöpperl, H., Schmidt, C. W., V., Dodd, J., Krumlauf, R., and Beddington, R. S. P. (1997) "Misexpression of *Cwnt8C* in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neurectoderm." Development (in

press).

- Rupp, R. A., Snider, L., and Weintraub, H. (1994) "Xenopus embryos regulate the nuclear localization of XMyoD." Genes Dev 8: 1311-23.
- 5 Sasaki, H., and Hogan, B. L. (1993) "Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo." Development 118: 47-59.
- 10 Sasaki, H., and Hogan, B. L. (1994) "HNF-3 beta as a regulator of floor plate development." Cell 76: 103-15.
- Slack, J. M. (1994) "Inducing factors in Xenopus early embryos." Curr. Biol. 4: 116-126.
- Sokol, S. Y. (1996) "Analysis of Dishevelled signalling pathways during *Xenopus* development." Curr. Biol. 6: 1456-1467.
- 15 St-Jacques, B., and McMahon, A. P. (1996) "Early mouse development: lessons from gene targeting." Curr. Opin. Genet. Dev. 6: 439-444.
- Sussman, D. J., Klingensmith, J., Salinas, P., Adams, P. S., Nusse, R., and Perrimon, N. (1994) "Isolation and characterization of a mouse homolog of the *Drosophila* segment polarity gene dishevelled." Devel. Biol. 166: 73-86.
- 20 Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K., and Ueno, N. (1994) "A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early *Xenopus* embryo." Proc Natl Acad Sci U S A 91: 10255-10259.
- 25 Tilghman, S. M. (1996) "Lessons learned, promises kept: a biologist's eye view of the Genome Project." Genome Res 6: 773-80.
- 30 Watabe, T., Kim, S., Candia, A., Rothbacher, U., Hashimoto, C., Inoue, K., and Cho, K. W. (1995) "Molecular mechanisms of Spemann's organizer formation: conserved growth factor synergy between *Xenopus* and mouse." Genes Dev 9: 3038-3050.
- 35 Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) "RGS family members: GTPase-

-51-

- activating proteins for heterotrimeric G-protein α -subunits." Nature 383: 172-175.
- Wilkinson, D. G. (1992) "Whole mount in situ hybridization of vertebrate embryos." In In situ hybridization: a practical approach, D. G. Wilkinson, ed. (Oxford: IRL Press), pp. 75-83.
- Wylie, C., Kofron, M., Payne, C., Anderson, R., Hosobuchi, M., Joseph, E., and Heasman, J. (1996) "Maternal beta-catenin establishes a 'dorsal signal' in early *Xenopus* embryos." Development 122: 2987-2996.
- Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J., and Nusse, R. (1995) "The dishevelled protein is modified by wingless signaling in *Drosophila*." Genes Dev 9: 1087-1097.
- Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D., and Moon, R. T. (1996) "The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3." Genes Dev. 10: 1443-1454.
- Ziv, T., Shimoni, Y., and Mitrani, E. (1992) "Activin can generate ectopic axial structures in chick blastoderm explants." Development 115: 689-694.

SECOND SECTION

Wnts are a family of secreted polypeptides which play many functions in development and tumorigenesis (Nusse, 1992; 5 Nusse and Varmus, 1992). The earliest Wnt genes to be identified were shown to have oncogenic effects in the mouse mammary gland. Wnt-1 was first identified as a target for activation by insertion of a retrovirus in mammary tumors ((Nusse, 1992; Nusse and Varmus, 1992) and references 10 therein), and inappropriate expression of Wnt-1 can also cause mammary gland tumorigenesis in transgenic mice (Tsukamoto et al., 1988). Wnts are believed to utilize a signal transduction pathway including the following components: The receptors for Wnts are believed to be 15 members of the frizzled family (Bhanot et al., 1996; Chan et al., 199; Wang et al., 1996; Yang-Snyder et al., 1996). The next known component of the pathway is Dishevelled (DSH) (Noordermeer et al., 1994), a cytoplasmic protein that is phosphorylated in response to wingless (Yanagawa et al., 20 1995). Through an unknown mechanism, Dsh inhibits the activity of glycogen synthase kinase-3 (GSK-3). In the absence of a Wnt signal, GSK-3 activity leads (directly or indirectly) to the phosphorylation and consequent degradation of β -catenin. In the presence of a Wnt signal, 25 GSK-3 is inhibited, increasing the cytosolic level of β -catenin, and promoting its interaction with downstream effectors (Behrens et al., 1996; Funayama et al., 1995; Molenaar et al., 1996; Yost et al., 1996). The product of the APC (adenomatous polyposis coli) gene is also involved 30 in Wnt signaling, apparently forming a complex with GSK-3 and β -catenin (Munemitsu et al., 1995; Papkoff et al., 1996; Polakis, 1997; Rubin et al., 1996; Rubinfeld et al., 1993).

35 Mutations in at least two components of the Wnt signaling pathway, APC, and β -catenin, have been associated with a variety of cancers, including hereditary colorectal cancer (Familial Adenomatous Polyposis coli, of FAP) and melanoma (Morin et al., 1997; Peifer, 1997; Polakis, 1997; Rubinfeld

-53-

et al., 1997). While colon cancer is the primary manifestation of FAP, some patients also develop tumors in other sites, such as other regions of the GI tract, in the thyroid, brain and abdominal cavity (Polakis, 1997). The role of APC in tumorigenesis is thought to be mediated by β -catenin. Normally, APC together with GSK-3 regulates the levels of free cytosolic β -catenin, which remain low. When APC is mutated, degradation of β -catenin is disrupted and levels of β -catenin are greatly increased (Peifer, 1997; Polakis, 1997). In addition, mutations in the β -catenin that interfere with its normal regulation have been found in cases of colon cancer (Morin et al., 1997) and melanoma (Rubinfeld et al., 1997).

Axin has been shown to negatively regulate signaling through the Wnt pathway, based on its ability to block induction of a secondary embryonic axis in frog embryos, when co-injected with mRNAs for Wnt, dishevelled or a dominant-negative mutant form of GSK-3 (Zeng et al., 1997). Axin cannot block axis duplication caused by injection of mRNA for β -catenin, indicating that it functions upstream of β -catenin, but downstream of GSK-3. Therefore, Axin is likely to promote the degradation of β -catenin, either directly or indirectly. Overexpression of Axin in pre-cancerous or tumor cells may be expected to counteract the effects of mutations in APC, β -catenin or other associated proteins, which would otherwise increase the levels of cytosolic β -catenin leading to cellular transformation. Therefore, Axin may be useful clinically to counteract the effects of such mutations. In addition, mutations in the Axin gene itself may result in loss of regulation of β -catenin levels and consequent tumorigenesis, i.e., Axin may be a tumor suppressor gene. Such mutations could either be loss-of-function mutations, or dominant-negative mutations. The ability of one such mutation (a deletion of the RGS domain of Axin) to create a dominant negative mutant form of the protein has been demonstrated (Zeng et al., 1997).

REFERENCES FOR THE SECOND SECTION

- Behrens, J., von Kries, J.P., Kuhl, M., Bruhn, L., Wedlich,
D., Grosschedl, R., and Birchmeier, W. (1996) "Functional
5 interaction of beta-catenin with the transcription factor
LEF-1, Nature 382: 638-642.
- Bhanot, P., Brink, M., Samos, C.H., Hesieh, J.C., Wang, Y.,
Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996)
"A new member of the frizzled family from Drosophila
10 functions as a Wingless receptor. Nature 382: 225-230.
- Chan, S.D., Karpf, D.B., Fowlkes, M.E., Hooks, M., Bradley,
M.S., Vuong, V., Bambino, T., Liu, M.Y., Arnaud, C.D.,
Strewler, G.J., and et al. (1992) "Two homologs of the
Drosophila polarity gene frizzled (fz) are widely
15 expressed in mammalian tissues. J. Biol. Chem 267: 25202-
25207.
- Funayama, N., Fagotto, F., McCrea, P., and Gumbiner, B.M.
(1995) "Embryonic axis induction by the armadillo repeat
domain of beta-catenin: evidence for intercellular
20 signaling." J. Cell. Biol. 128: 959-968.
- Molenaar, M., van de Wetering, M., Oosterwegel, M.,
Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J.,
Destree, O., and Clevers, H. (1996) "Xtcf-3 transcription
factor mediates beta-catenin-induced axis formation in
25 *Xenopus* embryos. Cell 86: 391-399.
- Morin, P.J., Sparks, A.B., Korinek, V., Barker, N.,
Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997)
"Activation of beta-catenin-Tcf signaling in colon cancer
by mutations in beta-catenin or APC [see comments].
30 Science 275: 1787-1790.
- Munemitsu, S., Albert, I., Souza, B., Rubinfeld, B., and
Polakis, P., (1995). "Regulation of intracellular beta-
catenin levels by the adenomatous polyposis coli (APC)
tumor-suppressor protein. Proc. Natl. Acad. Sci. U.S.A.
35 92: 3046-3050.
- Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse,
R. (1994) "Dishevelled and armadillo act in the wingless

-55-

- signaling pathway in *Drosophila*. Nature 367: 80-83.
- Nusse, R. (1992) "The Wnt gene family in tumorigenesis and in normal development [Review]. J. Steroid Biochem. Mol. Biol. 43: 9-12.
- 5 Nusse, R., and Varmus, H.E. (1992) "Wnt genes" Cell 69: 1073-1087.
- Papkoff, J., Rubinfeld, B., Schryver, B., and Polakis, P. (1996) "Wnt-1 regulates free pools of catenins and stabilizes APC-catenin complexes." Mol. Cell. Biol. 16: 2128-2134.
- 10 Peifer, M., (1997) "Beta-catenin as oncogene: the smoking gun." Science 275: 1752-1753.
- Polakis, P. (1997) "The adenomatous polyposis coli (APC) tumor suppressor." Biochim. Biophys. Acta. 1332: F127-147.
- 15 Rubinfeld, B., Albert, I., Porfiri, E., Fiol, C., Munemitsu, S., and Polakis, P. (1996) "Binding of GSK3 β to the APC- β -catenin complex and regulation of complex assembly." Science 272: 1023-1026.
- Rubinfeld, B., Robbins, P., El-Gamil, M., Albert, I., Porfiri, E., and Polakis, P. (1997) "Stabilization of beta-catenin by genetic defects in melanoma cell lines." Science 275: 1790-1792.
- 20 Rubinfeld, B., Souza, B., Albert, I., Muller, O., Chamberlain, S.H., Masiarz, F.R., Munemitsu, S., and Polakis, P. (1993) "Association of the APC gene product with beta-catenin." Science 262: 1731-1734.
- 25 Tsukamoto, A.S., Grosschedl, R., Guzman, R.C., Parslow, T., and Varmus, H.E. (1988) "Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice." Cell 55: 619-625.
- 30 Wang, Y., Macke, J.P., Abella, B.S., Andreasson, K., Worley, P., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., and Nathans, J., (1996) "A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene *frizzled*." J. Biol. Chem. 271: 4468-4476.
- 35 Yanagawa, S., van Leeuwen, F., Wodarz, A., Klingensmith, J.,

-56-

- and Nusse, R. (1995) "The dishevelled protein is modified by wingless signaling in *Drosophila*." Genes Dev. 9: 1087-1097.
- 5 Yang-Synder, J., Miller, J.R., Brown, J.D., Lai, C.J., and Moon, R.T. (1996) "A frizzled homolog functions in a vertebrate *Wnt* signaling pathway." Curr. Biol. 6: 1302-1306.
- 10 Yost, C., Torres, M., Miller, J.R., Huang, E., Kimelman, D., and Moon, R.T. (1996) "The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3." Genes Dev. 10: 1443-1454.
- 15 Zeng, L., Fagotto, F., Zhang, T., Hsu, W., Vasicek, T.J., III, P.W.L., Lee, J.J., Tilghman, S.M., Gumbiner, B.M., and Costantini, F. (1997) "The mouse *Fused* locus encodes Axin, an inhibitor of the *Wnt* signaling pathway that regulates embryonic axis formation." Cell 90: 181-192.

THIRD SERIES OF EXPERIMENTS

The previous series of experiments concluded that Axin could influence a signal transduction pathway that includes the proteins Wnt (members of the Wnt family of proteins), dishevelled, GSK-3, APC and β -catenin. This conclusion was based on experiments in frog embryos, which showed that Axin mRNA injected into the frog embryo could inhibit the ability of several of these proteins to induce an embryonic axis (Zeng et al., 1997, *Cell* 90, 181-192).

Additional biochemical and genetic data support the involvement of Axin in this signaling pathway. Forms of the Axin protein that can bind directly to APC, GSK-3 and β -catenin were found through a number of assays. Binding of Axin to GSK-3 and to β -catenin was demonstrated by biochemical binding experiments in vitro, as well as by co-immunoprecipitation studies. The former type of experiment shows that highly purified preparations of the proteins (e.g., Axin and GSK-3, or Axin and β -catenin) have the capacity to bind directly to each other. The latter type of experiment shows that, in living mammalian cells, the two proteins are present as part of the same protein complex, so that when one is specifically "pulled out" of a cell lysate, the other protein comes along with it. The binding to APC was shown by both of these methods, as well as by a method known as the yeast two-hybrid system (Fields, S. & Song, 1989, *O. Nature* 340, 245-6).

The ability of Axin to bind to these three proteins supports the contention that Axin is a regulator of signaling through this pathway, and suggests a mechanism by which it may function. Since Axin can bind to both GSK-3 and β -catenin, it could stimulate the ability of GSK-3, a serine-threonine kinase, to phosphorylate β -catenin, which is believed to lead to the degradation of the latter protein. β -catenin that escapes degradation is thought to convey the signal, which in some cells leads to cellular transformation and

-58-

cancer. By promoting the degradation of β -catenin, Axin, or other molecules that interact with Axin, could inhibit signaling through β -catenin, and thus inhibit cellular transformation (oncogenic transformation, i.e. from a normal cell to a cancer cell) and tumorigenesis. Thus, Axin could act as a "tumor supressor".

What is claimed is:

1. An isolated nucleic acid which encodes Axin.
- 5 2. The isolated nucleic acid of claim 1, wherein the nucleic acid is DNA or RNA
3. The isolated nucleic acid of claim 2, wherein the nucleic acid is cDNA or genomic DNA.
- 10 4. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes mutant Axin.
5. The isolated nucleic acid of claim 1, wherein the
15 nucleic acid encodes wildtype Axin.
6. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes a mouse wildtype Axin.
- 20 7. The isolated nucleic acid of claim 1 comprising a nucleic acid having the sequence designated Seq. I.D. No.: 2.
8. The isolated nucleic acid of claim 1, wherein the
25 nucleic acid encodes a mouse wildtype having substantially the same amino acid sequence as the sequence designated Seq. I.D. No.: 1.
9. An isolated nucleic acid which encodes a polypeptide
30 comprising the amino acid sequence of Axin.
10. A vector comprising the isolated nucleic acid of claim 1.
- 35 11. The vector of claim 10, further comprising a promoter of RNA transcription operatively linked to the nucleic acid.

-60-

12. The vector of claim 10, wherein the promoter comprises a bacterial, yeast, insect or mammalian promoter.
13. The vector of claim 10, further comprising plasmid, cosmid, yeast artificial chromosome (YAC), bacteriophage or eukaryotic viral DNA.
14. The cosmid of claim 13 designated GenBank Accession No. Z69667.
15. The cosmid of claim 13, designated GenBank Accession No. Z81450.
16. A host vector system for the production of a polypeptide which comprises the vector of claim 10 in a suitable host.
17. The host vector system of claim 16, wherein the suitable host is a prokaryotic or eukaryotic cell.
18. The host vector system of claim 17, wherein the prokaryotic cell is a bacterial cell.
19. The host vector system of claim 17, wherein the eukaryotic cell is a yeast, insect, plant or mammalian cell.
20. A method for producing a polypeptide which comprises growing the host vector system of claim 16 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
21. A method of obtaining a polypeptide in purified form which comprises:
- (a) introducing the vector of claim 10 into a suitable host cell;
 - (b) culturing the resulting cell so as to produce the polypeptide;

-61-

- (c) recovering the polypeptide produced in step (b);
and
 - (d) purifying the polypeptide so recovered.
- 5 22. A polypeptide comprising the amino acid sequence of Axin.
23. A purified wildtype Axin.
- 10 24. A purified mutant Axin.
25. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes wildtype Axin without hybridizing to a nucleic acid which encodes mutant Axin.
- 15
26. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within the nucleic acid which encodes mutant Axin without hybridizing to a nucleic acid which encodes wildtype Axin.
- 20
27. The oligonucleotide of claim 25 or 26, wherein the nucleic acid is DNA or RNA.
- 25
28. A nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid of claim 1.
- 30 29. An antisense molecule capable of specifically hybridizing with the isolated nucleic acid of claim 4.
30. A method for determining whether a subject carries a mutation in the axin gene which comprises:
- 35 (a) obtaining an appropriate nucleic acid sample from the subject; and
- (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid

-62-

which encodes mutant Axin so as to thereby determine whether a subject carries a mutation in the axin gene.

- 5 31. The method of claim 30, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein the determining of step (b) comprises:
- 10 (i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;
- (ii) isolating the complex so formed; and
- 15 (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.
- 20 32. The method of claim 30, wherein the determining of step (b) comprises:
- (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into
- 25 distinct, distinguishable pieces of nucleic acid;
- (ii) isolating the pieces of nucleic acid; and
- 30 (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes
- 35 mutant Axin.
33. The method of claim 30, wherein the determining of step (b) comprises:

-63-

- (i) sequencing the nucleic acid sample of step (a); and
- (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.
34. The method of claim 30, wherein the determining of step (b) comprises:
- (i) amplifying the nucleic acid present in the sample of step (a); and
- (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.
35. The method of claim 31, 32 or 33, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.
36. The method the claim 35, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.
37. The method of claim 30, wherein the sample comprises blood, tissues or sera.
38. A method for determining whether a subject has a predisposition for cancer which comprises:
- (a) obtaining an appropriate nucleic acid sample from the subject; and
- (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has a predisposition for cancer.
39. The method of claim 38, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant Axin, and wherein

-64-

the determining of step (b) comprises:

- (i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;
- (ii) isolating the complex so formed; and
- (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

40. The method of claim 38, wherein the determining of step (b) comprises:

- (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;
- (ii) isolating the pieces of nucleic acid; and
- (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

41. The method of claim 38, wherein the determining of step (b) comprises:

- (i) sequencing the nucleic acid sample of step (a); and
- (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.

-65-

42. The method of claim 38, wherein the determining of step (b) comprises:
- (i) amplifying the nucleic acid present in the sample of step (a); and
 - 5 (ii) detecting the presence of the mutant Axin in the resulting amplified nucleic acid.
43. The method of claim 39, 40 or 41, wherein the isolated nucleic acid or the oligonucleotide is labeled with a
10 detectable marker.
44. The method the claim 43, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.
- 15 45. The method of claim 38, wherein the sample comprises blood, tissues or sera.
46. The method of claim 38, wherein the cancer comprises breast, colorectal, gastrointestinal, esophageal
20 carcinomas or melanomas.
47. A method for treating a subject who has a predisposition to cancer which comprises introducing the isolated nucleic acid of claim 5 into the subject
25 under conditions such that the nucleic acid expresses wildtype Axin, so as to thereby treat the subject.
48. A method for treating a subject who has a predisposition to cancer which comprises an effective
30 amount of the wildtype human homolog of Axin and a pharmaceutically acceptable carrier, so as to thereby treat the subject who is susceptible to cancer.
49. The method of claim 47 or 48, wherein the cancer
35 comprises brain, breast, colorectal, gastrointestinal, esophageal or thyroid carcinomas.
50. A method for determining whether a subject has cancer,

-66-

which comprises:

- (a) obtaining an appropriate nucleic acid sample from the subject; and
- (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby determine whether a subject has cancer.

51. The method of claim 50, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding mutant Axin, and wherein the determining of step (b) comprises:

- (i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;
- (ii) isolating the complex so formed; and
- (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.

52. The method of claim 50, wherein the determining of step (b) comprises:

- (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;
- (ii) isolating the pieces of nucleic acid; and
- (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes

-67-

mutant Axin.

53. The method of claim 50, wherein the determining of step (b) comprises:
- 5 (i) sequencing the nucleic acid sample of step (a); and
- (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the
- 10 nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.
54. The method of claim 50, wherein the determining of step (b) comprises:
- 15 (i) amplifying the nucleic acid present in the sample of step (a); and
- (ii) detecting the presence of mutant Axin in the resulting amplified nucleic acid.
- 20 55. The method of claim 51, 52 or 53, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.
56. The method the claim 55, wherein the detectable marker
- 25 is a radioactive isotope, a fluorophor or an enzyme.
57. The method of claim 50, wherein the sample comprises blood, tissue or sera.
- 30 58. The method of claim 50, wherein the cancer comprises brain, breast, colorectal, gastrointestinal, esophageal, thyroid carcinomas or melanomas.
59. A method for detecting a mutation in cancerous cells of
- 35 the subject which comprises:
- (a) obtaining an appropriate nucleic acid sample from the subject; and
- (b) determining whether the nucleic acid sample from

-68-

step (a) is, or is derived from, a nucleic acid which encodes mutant Axin so as to thereby detect a mutation in the cancerous cells of the subject.

- 5 60. The method of claim 59, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding mutant Axin, and wherein the determining of step (b) comprises:
- 10 (i) contacting the mRNA with the oligonucleotide of claim 26 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;
- (ii) isolating the complex so formed; and
- 15 (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant Axin.
- 20 61. The method of claim 59, wherein the determining of step (b) comprises:
- (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into
- 25 distinct, distinguishable pieces of nucleic acid;
- (ii) isolating the pieces of nucleic acid; and
- 30 (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes
- 35 mutant Axin.
62. The method of claim 59, wherein the determining of step (b) comprises:

-69-

- (i) sequencing the nucleic acid sample of step (a); and
- (ii) comparing the nucleic acid sequence of step (i) with the isolated nucleic acid of claim 5, so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant Axin.
63. The method of claim 59, wherein the determining of step (b) comprises:
- (i) amplifying the nucleic acid present in the sample of step (a); and
- (ii) detecting the presence of mutant Axin in the resulting amplified nucleic acid.
64. The method of claim 61, 62 or 63, wherein the isolated nucleic acid or the oligonucleotide is labeled with a detectable marker.
65. The method the claim 64, wherein the detectable marker is a radioactive isotope, a fluorophor or an enzyme.
66. The method of claim 59, wherein the sample comprises blood, tissue or sera.
67. The method of claim 59, wherein the cancer comprises brain, breast, colorectal, gastrointestinal, esophageal, thryoid carcinomas or melanomas.
68. A method of suppressing cells unable to regulate themselves which comprises introducing the isolated nucleic acid of claim 5 into the cells.
69. The method of claim 68, wherein introducing the isolated nucleic acid, comprises:
- (a) recovering cells unable to regulate themselves from the subject;
- (b) introducing the isolated nucleic acid of claim 5

-70-

into the cells; and

(c) reintroducing the cells of step (b) into the subject so as to suppress those cells.

5 70. The method of claim 68, wherein the cells are cancerous.

71. The method of claim 70, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, skin,
10 colon, rectum, esophagus, stomach, thyroid or intestine.

72. The method of claim 68, wherein the cells are unable to regulate themselves because of a mutation in the axin
15 gene.

73. The method of claim 68, wherein the cells are unable to regulate themselves of a mutation in the adenomatous polyposis coli gene.
20

74. A method of suppressing cells unable to regulate themselves which comprises introducing wildtype Axin into the cells in an amount effective enough to suppress the cells.
25

75. The method of claim 74, wherein the cells are cancerous.

76. The method of claim 75, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, skin,
30 colon, rectum, esophagus, stomach, thyroid or intestine.

77. The method of claim 68, wherein the cells are unable to regulate themselves because of a mutation in the axin
35 gene.

78. The method of claim 68, wherein the cells are unable to

-71-

regulate themselves of a mutation in the adenomatous polyposis coli gene.

- 5 79. A method of treating a subject who has cancer which comprises introducing the isolated nucleic acid of claim 5, into the subject so as to thereby treat the cancer.
- 10 80. The method of claim 79, wherein introducing the isolated nucleic acid, comprises:
(a) recovering cancerous cells from the subject;
(b) introducing the isolated nucleic acid of claim 5 into the cells; and
15 (c) reintroducing the cells of step (b) into the subject so as to treat the subject who has cancer.
- 20 81. The method of claim 80, wherein the cancerous cells are derived from the abdominal cavity, brain, breast, colon, skin, rectum, esophagus, stomach, thyroid or intestine.
- 25 82. A method for identifying a chemical compound which is capable of suppressing cells unable to regulate themselves in a subject which comprises:
(a) contacting mutant Axin with the chemical compound under conditions permitting binding between the mutant Axin and the chemical compound;
(b) detecting specific binding of the chemical compound to the mutant Axin; and
30 (c) determining whether the chemical compound inhibits the mutant Axin so as to identify a chemical compound which is capable of suppressing cells unable to regulate themselves.
- 35 83. The method of claim 82, wherein the cells are cancerous.
84. The method of claim 83, wherein the cancerous cells are

-72-

derived from the abdominal cavity, brain, breast, skin, colon, rectum, esophagus, stomach, thyroid or intestine..

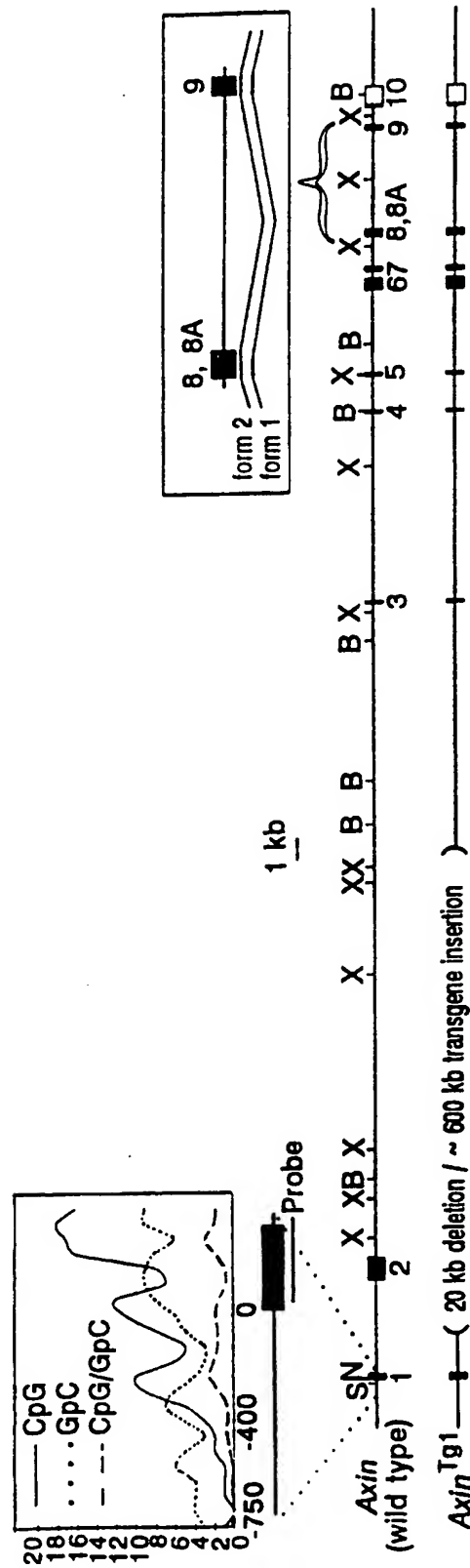
- 5 85. A pharmaceutical composition comprising the chemical compound identified by the method of claim 82 in an amount effective to inhibit cancer and a pharmaceutically effective carrier.
- 10 86. A pharmaceutical compositions comprising the antisense molecule of claim 25 in an amount effective to treat cancer and a pharmaceutically effective carrier.
- 15 87. A pharmaceutical composition comprising the purified wildtype Axin in an amount effective to treat cancer and a pharmaceutically effective carrier.
- 20 88. A method of treating a subject who has cancer comprising administration of an effective amount of the pharmaceutical composition of claim 85, 86 or 87 to the subject who has cancer.
- 25 89. The method of claim 88, wherein the administration comprises, topical, oral, aerosol, subcutaneous administration, infusion, intralesional, intramuscular, intraperitoneal, intratumoral, intratracheal, intravenous injection, or liposome-mediate delivery.
- 30 90. A transgenic, nonhuman mammal comprising the isolated nucleic acid of claim 1.
91. The transgenic, nonhuman mammal of claim 90, wherein the nucleic acid encodes mutant Axin.
- 35 92. The method of claim 47, wherein the nucleic acid is overexpressed so as to thereby treat the subject.
93. A method of inhibiting tumorigenesis in a subject which

-73-

comprises administering an effective amount of a molecule which induces degradation of β -catenin thereby inhibiting tumorigensis.

- 5 94. The method of claim 94, wherein the molecule is Axin or a functionally active mutant of Axin.
95. The method of claim 94, wherein the molecule is a molecule which inhibits an enzyme.
- 10 96. A method of treating a subject with cancer which comprises administering an effective amount of a pharmaceutical composition comprising a molecule which induces degradation of β -catenin and a pharmaceutically acceptable carrier, thereby treating the subject with cancer.
- 15
97. The method of claim 96, wherein the molecule which induces degradation of β -catenin is Axin or a functionally active mutant of Axin.
- 20
98. The method of claim 96, wherein the molecule is a molecule which inhibits an enzyme.

FIG. 1



2/19

FIG. 2A

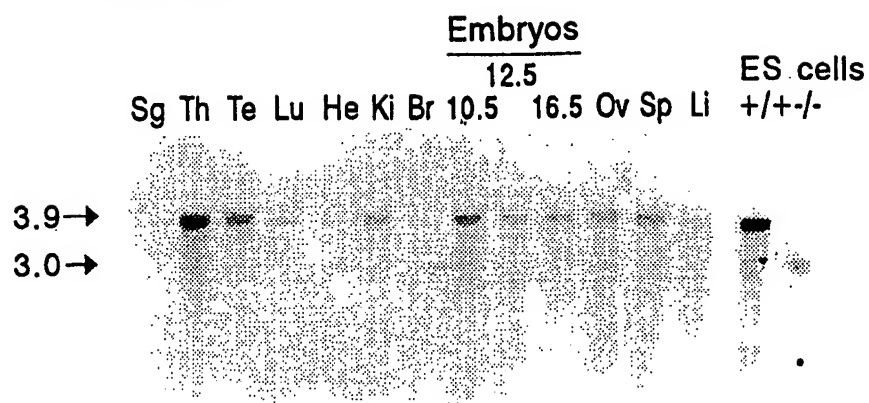


FIG. 2B



FIG. 2C

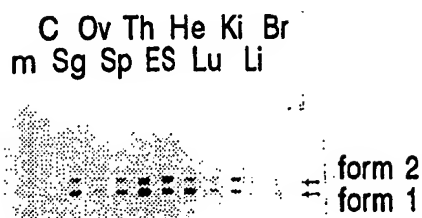
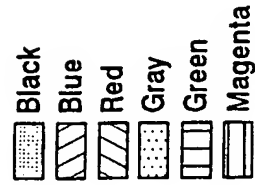


FIG. 3A

m	1	LGSGSRLREALAAAAAGACWGRAGAWFQRGLVRVSSRFWRRSAACLAPPPGHGSPSRRRR
m	61	RDGGPPGPRRRGPPAEPLSAWASPGREP
h		1 GPGSRH - RARRLHFGAVSTDLGCSA
c		1 DFGSSP
m	121	HCSLMQSPKMNVEQGGFPLDLGASFTEDAPRPVPGEEGELVSTDSPVNHSCSGKGT
h	29	HCSLTQSPKMNVEQGGFPLDLGASFTEDAPRPVPGEEGELVSTDSPVNHSCSGKGT
c	7	AVQTSAR-KMNIQGGKGFPLDLGRSFTEDAPRPVPGEEGELVSTDSPVNHSCSGKGT
m	181	IKSETSTATPRRSDLDLGYEPEGSASPTPPYLIRWAESLHSLDDQDGI
h	89	IKSETSTATPRRSDLDLGYEPEGSASPTPPYLIRWAESLHSLDDQDGI
c	66	VHNETSTATPRRSDLDLGYEPEGSASPTPPYLIRWAESLHSLDDQDGI
m	241	ADLLDFWACSGFRKLEPCDSNEEKRLKLARATVRKYILDNGIVSRQTKPATKSF
h	149	ADLLDFWACSGFRKLEPCDSNEEKRLKLARATVRKYILDNGIVSRQTKPATKSF
c	126	ADLLDFWACSGFRKLEPCDSNEEKRLKLARATVRKYILDNGIVSRQTKPATKSF
m	301	VMKQIDRAMFDCAQTEIQSTMEENTYPSFLKSDIYLEVTRTGSESPKVCSDQSSSGTG
h	209	VMKQIDRAMFDCAQTEIQSTMEENTYPSFLKSDIYLEVTRTGSESPKVCSDQSSSGTG
c	186	VMKQIDRAMFDCAQTEIQSTMEENTYPSFLKSDIYLEVTRTGSESPKVCSDQSSSGTG
m	361	KGMGSLPTLNEDEEWKCDQDADDEDDGRDP LPPSRLTQKLLLETAAPRA
h	269	KGMGSLPTLNEDEEWKCDQDADDEDDGRDP LPPSRLTQKLLLETAAPRA
c	246	KGMGSLPTLNEDEEWKCDQDADDEDDGRDP LPPSRLTQKLLLETAAPRA
m	421	FRYGSWREPVPYVNSGYALAPATSANDSEQQSSDADTSLTIDSSVDGIPPYRIRKQ
h	329	FRYGSWREPVPYVNSGYALAPATSANDSEQQSSDADTSLTIDSSVDGIPPYRIRKQ
c	306	FRYGSWREPVPYVNSGYALAPATSANDSEQQSSDADTSLTIDSSVDGIPPYRIRKQ
m	481	HRREMQUESIQVNGRVPLPHIPRTYRMPKEIRVEPQKFAEELIHRLEAVQRTREAEKLEE
h	389	HRREMQUESIQVNGRVPLPHIPRTYRMPKEIRVEPQKFAEELIHRLEAVQRTREAEKLEE
c	366	HRREMQUESIQVNGRVPLPHIPRTYRMPKEIRVEPQKFAEELIHRLEAVQRTREAEKLEE



4/19

FIG. 3B

m	541	RLKRVRMEEEGEDGEMPSSGR - MASHKLPSVPAWHHFPTRY - VDMGCSGL - - - RDAHEENPE
h	449	RLKRVRMEEEGEDGDPSSGPPGPCHKLFPAPAWHHFPRLCWTWACAGL - - - RDAHEENPE
c	426	RLKRVRAEEEGEDADISSGPSMISHKMPSAQPFHHPAPRY - SEMGCAGMQMRDAHEENPE
m	597	SILDEHVQRVMRTPGCQSPGPG - H - - - RSPDSGHVAKT - AVLGGTASGHGKHVPKGLK
h	507	SILDEHVQRVLERTTGRQSPGPG - H - - - RSPDSGHVAKMPVALGGAASGHGKHVPKSGAK
c	485	SILDEHVQRVMKTPGCQSPGPGRHSPKPRSPESGHLGKLSGTLGTIPRGGHGHKHTTKSGMK
m	651	LDIAGLHHHRHVHHVHHNSAR - PKEQMEAEVARRVQSSF - SWGPETHGHAK - PRSYSENA
h	562	LDAAGLHHHRHVHHVHHSTAR - PKEQVEAEATRRAQSSF - AWGLEPESHGARSRGYSES
c	545	LDAANLYHHRHVHHVHHHSMMKPKQIEAEATQRVQNSF - AWWVDSHNYATKSRNYSEN
m	709	GITLSAGD - LAFGGKTSAPSKRNTKKAESGKN - - - ANAEVPSTTEDAEKNQKIMQWIIIEGE
h	621	GAPNMSDGLAFSGKVGMAKRNKKAESGKS - - - ASTEVPGASEDAEKNQKIMQWIIIEGE
c	605	GMAPVMDSLGYSKGKASLISKRNIKKTDGKSDGANVEMP - GSPEDVERNQKILQWIIIEGE
m	766	KEISRHRKAGHGSSGLRKQQAHESSRPLSIERPDAVHPWVSAQLRNSVQPSHLEIQDPTM
h	679	KEISRHRRTGHGSSGTRKPPHENSRLSLE - - - -HPWAGPQLRTSVQPSHLEIQDPTM
c	665	KEISRHRKTNHGSSGVKKQLSHDMVRPLSIERPMAVHPWVSAQLRNVQPSHPFIQDPTM
m	826	PPNPAPNPLTQLEEARRRLEEEEEKRANKLPSKQRYVQAVMQGRITCVRPACAPVLSVPA
h	734	PPHPAPNPLTQLEEARRRLEEEEEKRASRAPSQRYVQEVMMRRGRACVRPACAPVLHVPA
c	725	PPNPAPNPLTQLEEARRRLEEEEEKRAGKLPKQR - - - 36aa insert in form 2 - -
m	886	VSDLELSETETKSQRKAGGGSAPPCDSIVVGYFCGEPIPYRTLVRGRAVTLGQFKELLT
h	794	VSDMELSETETRQRKVGGSQAQPCDSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELLT
c	759	-----LKPKKPPGSGASQPCENIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELLT
m	946	KKGSYRYFYFKKYSDEFDCGVVFFEEVREDEPVLPVFFEEKIY - GKVEKVD 992
h	854	KKGSYRYFYFKKYSDEFDCGVVFFEEVREDEAVLPVFFEEKIY - GKVEKVD 900
c	809	KKGNRYRYFYFKKYSDEFDCGVVFFEEVREDEPTILPVFFEEKIY - GKVEKID 855

FIG. 3C

hRGS2	LWSEAFDELLASKYGLAAERAFLLKSEFCEENIEFWLACEDIFKKTIKSP	---QKISSKARKIYTD		
hRGS5	QWRDSLDKLLQNNYGLASFSLKSEFSEENLEFWLACEDYKKTIKSP	---AKMAEKAKQIYEE		
hRGS3	KWGESLEKLLVHKYGLAVFQAFLLRTIEFSEENLEFWLACEDIFKKTIKSQ	---SKMASAKAKKIFAE		
rRGS4	KWAESLENLISHECGLAAFRALFKSEYSEENIDFWLSCHEEYKKTIKSP	---SKLSPKAKKIYNE		
hRGS1	QWSSLEKLLANQTGQNVFGSLKSEFSEENIEFWLACEDYKKTIKES	---DLLPKAKKEIYKA		
hGAIP	SWAGSFDKLMHSPAGRSVFRALRTIEYSEENMLFWLACHEELKAEANQ	---HVVDEKARLIYED		
hRGS7	RWGFQGMDEALKDPVGREQFLKLESEFSSSENLEFWLAVEDLKKRPI	---KEVPSRVQEIWQE		
hRGS10	KWAASLENLLEDPEGVKRFRIEFLKKEFSEENMLFWLACEDIFKKTQKD	---TQMDEKAKEIYMT		
mAxin	RWAESLHSLDDQDGISLFRITFLKQEGCADLLDFWFAQSGFRKLEPCDSNEEKRLKLARIAIYRK			
hRGS2	FTEKEA	---PKEINIDFQTKTLLIAGNIQEA	TSGCHTTAQKRVYSLMENN	SYPRFLESEFYQDL
hRGS5	FIIQTEA	---PKEVNIDHFTKDIIMKNLVEPISL	SSFDMAQKRTHALMEKDS	LPFRVSEFYQEL
hRGS3	YIAIQA	---CKEVLDSYTRHETJKDNLQSV	TRGCFDLAQKRITFGLMEKDSYPRFL	RSDLYLDL
rRGS4	FISVQA	---TKEVNLDSCTREETSRLMLEP	TIITCFDEAOKKIENLMEKDSYPRFL	KSRFYLDL
hRGS1	FMHSDA	---AKQINIDERTRESTAKKIKAP	TPTCFDEAOKVIYITLMEKDSYPRFL	KSHIYLN
hGAIP	YVSIIS	---PKEVSLDSRVREGINKKMG	PSAHTFDDAQLQIYITLMHRDSYPRFL	SSPTYRAL
hRGS7	FLJARGA	---PSAINLDSKSYDKITQNVKEPGRYTF	EDAQEHYKLMKDSYPRFL	IRSSAYQEL
hRGS10	FLSSKA	---SSQVNEGQ-SRLNEKILIEEPHPLMFQKLQDQI	FINLMEKDSYPRFL	KSDLFK
mAxin	YILDSNGIVSRQTKPATKSF	IKDCVMKQIQIDPAMFDDAQTE	IQSTIMEENTYPSF	FLKSDIYLEY

FIG. 3D

mDv1-1	28	VTLADFKNNVLSNRPVHAYKKEFFKSMDDQDF	--GVVKEEIFFDDNAKLPCFNGRVV	78
mDv1-2	38	ITLGDFFKSVLQRI--PAGAKYFFKSMDDQDF	--GVVKEEISDDNARLPCFNGRVV	86
Dsh	35	VTLRDFKLVLNKQ--NNNYKYFFKSMDDADF	--GVVKEEIADDSTILPCFNGRVV	84
mAxin	935	VTLGDFFKELLTK--KGSVRYFYFKVSDDEFDCGVVFEFVREDEPVL	LPVFEETII	985

6/19

FIG. 4A-1

Axin



FIG. 4A-2

Axin + Xwnt8



FIG. 4A-3

Axin + β -catenin



FIG. 4A-4

Axin + Siamois

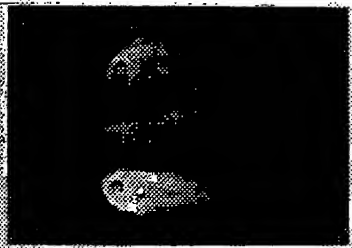


FIG. 4A-5

Control (β -galactosidase)



7/19

FIG. 4B

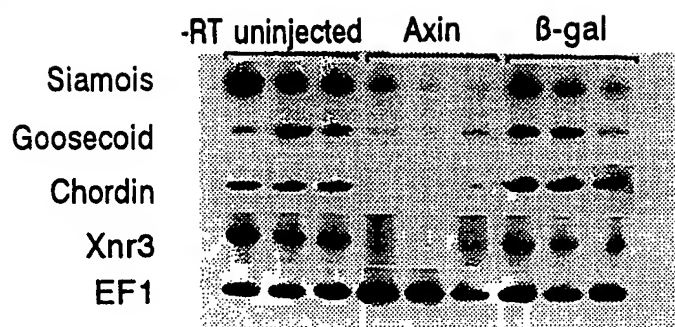
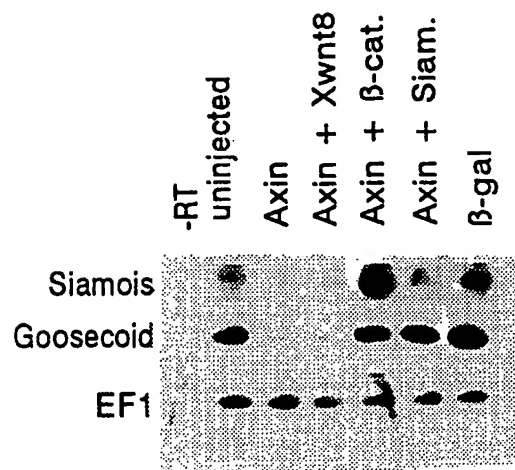
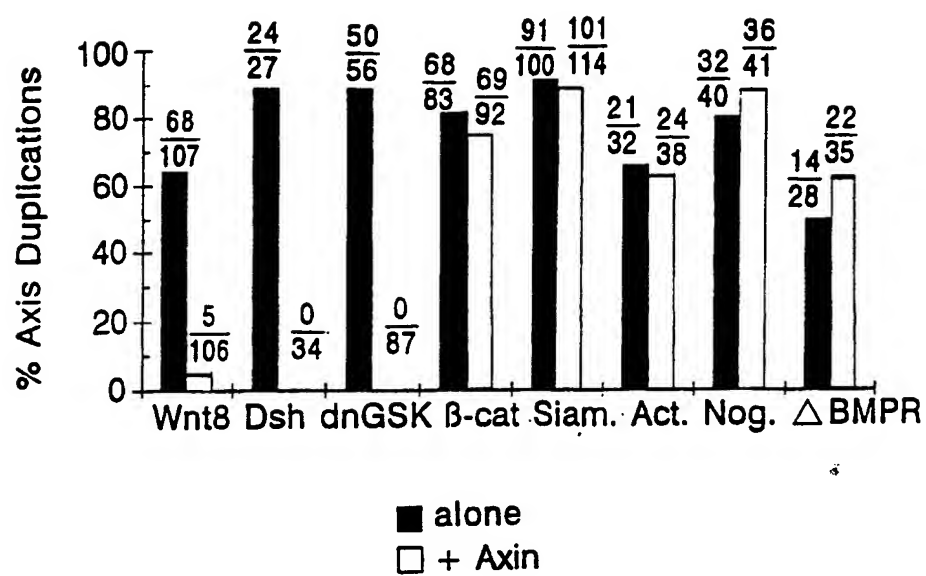


FIG. 4C



8/19

FIG. 5A



9/19

FIG. 5B-1 dnGSK-3



dnGSK-3 + Axin

FIG. 5B-2

FIG. 5B-3 β -catenin β -catenin + Axin

FIG. 5B-4



FIG. 5B-5 Siamois

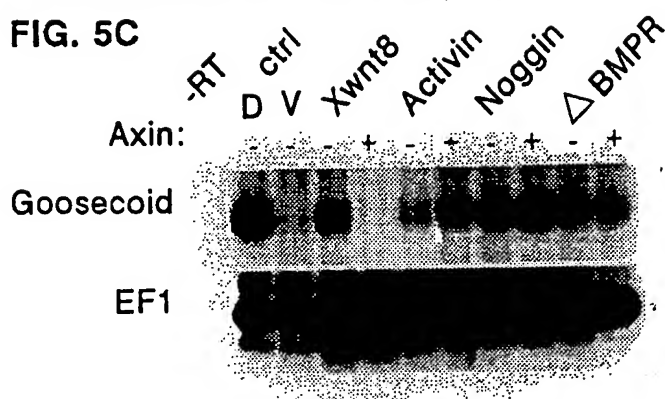


Siamois + Axin

FIG. 5B-6



FIG. 5C



10/19

FIG. 6A

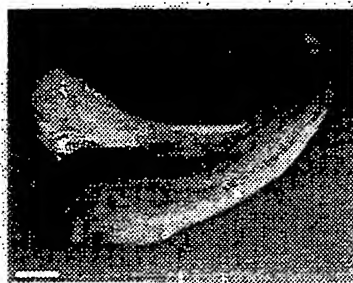
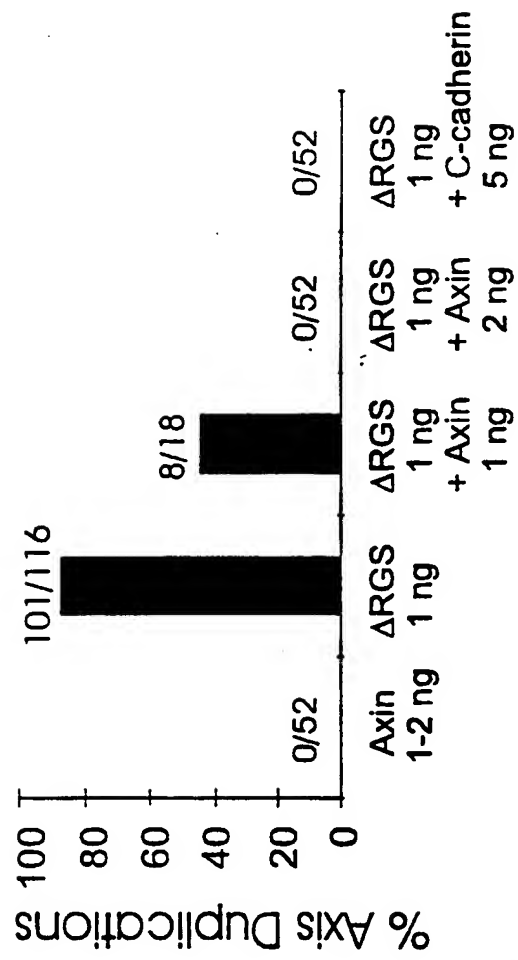


FIG. 6B



11/19

FIG. 6C



12/19

FIG. 6D

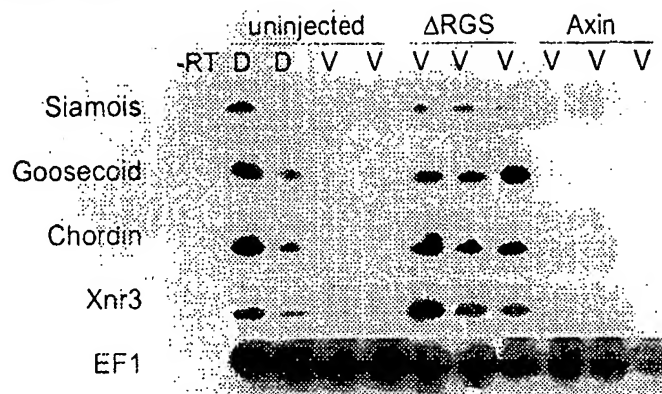


FIG. 6E

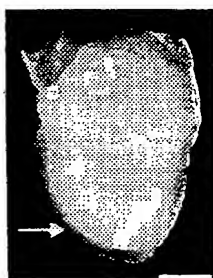
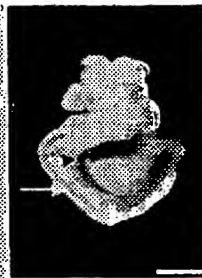


FIG. 6F

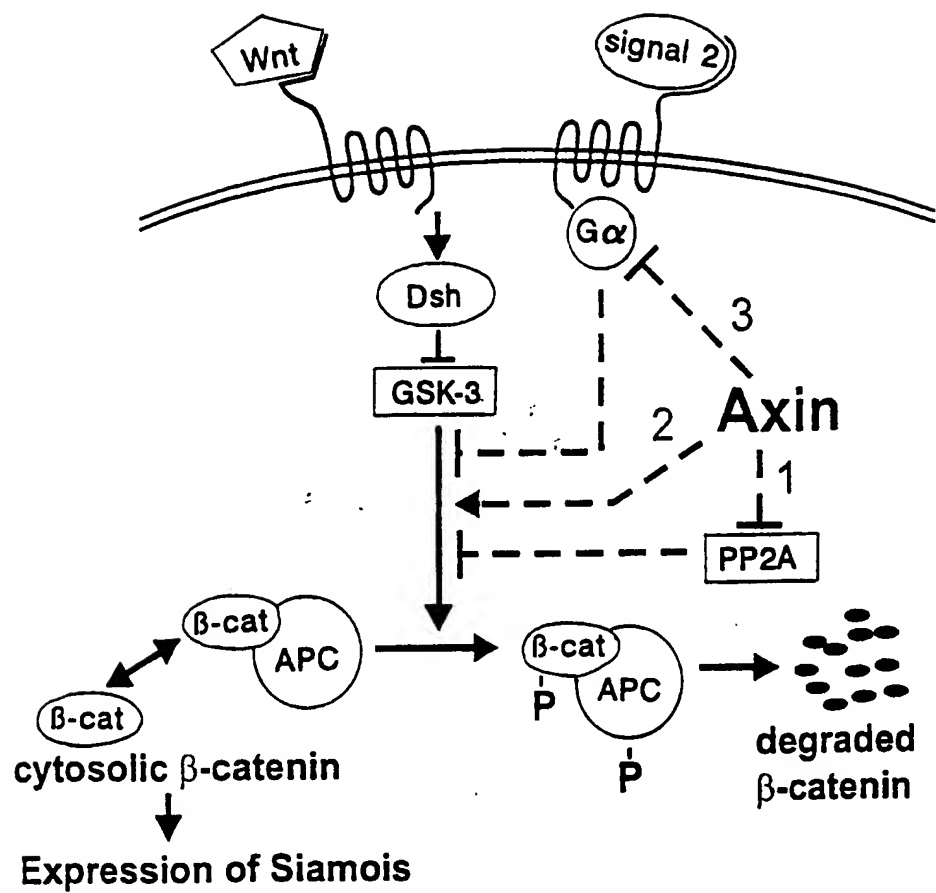


FIG. 6G



13/19

FIG. 7



14/19

FIG. 8

/translation="LGSGRLLREALAAAGACWGRAGAWFQGLVRVSSRFWRRSAA
CLAPPPGHGSPRRRRRDGPPGPRRRGPPAEPPLSAWASPGREP GPPRLHSRRAL
ERLIPLGAVSTEVLCSAHCSLMQSPKMNVEQGFPLDLGASFTEADAPRPPVPGE EGE
LVSTDSRPVNHSCGKGTSIKSETSTATPRRSDLDLGYEPEGASPTPPYLRWAESL
HSLDDQDGI SLFRFLKQEGCADLLDFWACSGFRKLEPCDSNEEKRLKLARAIYRK
YILDSNGIVSRQTKPATKSF IKDCVMKQOIDPAMFDQAQTEIQSTMEENTYPSFLKSD
IYLEYTRTGSESPKVCSDQSSSGTGKMSGYLPTLNEDEEWKCDQDADEDDGRDPLP
PSRLTQKLLLETAAPRAPSSRRYNEGRELRYGSWREPVPYVNSGYALAPATSANDS
EQQLSSDADTLSLTDSSVDGIPPYRI RKQHRREMQUESIQVNGRVP LPHIPRTYRMPK
EIRVEPQKFAEELIHRLEAVQRTREAEKLEERLKRVRMEEEGEDGEMPSGPMASHKL
PSVPAWHHFP PPRYVDMGCSGLRDAHEENPESILDEHVQRMRTPGCQSPGPGHRS PDS
GHVAKTAVLGGTASGHGKHVPKLGKLDTAGLHHHRHVHHVHNSARPKEQMEAEVA
RRVQSSFSWGPETHGHAKPRSYSENAGTTL SAGDLPFGGKTSAPSKRNTKKAESGKNA
NAEVPSTTEDAEKNQKIMQWII EGEKEISRHRKAGHGSSGLRKQQAHESSRPLSIERP
GAVHPWVSAQLRNSVQPSHLFIQDPTMPPNPAPNPLTQLEEARRRLEEEK RANKLPS
KQRYVQAVMQRGRTCVRPACAPVLSVVPVAVSDLELSETETKSQRKAGGSAPP CDSIV
VGYFFCGEPIPYRTLVRGRAVTLGQFKELLTKKGSYRYFFKKVSDEFDCGVVFEVRE
DEPVL PVFEEKIIGKVEKVD"

15/19

FIG. 9A

1 ctcttgggctc aggtccccgg ctacaggaag cgcttgccgc cgccgccgca ggagcctgct
61 61 ggggtcgggc tggagcctgg ttccagagag ggctggtgag agtgagcagc cggttttggc
121 121 ggcgtctcgc ggcctgcctt ggcgcgcctc gggccacgg gtcgccgagc cgccgccgcc
181 181 gccgcgatgg gggcccccca gggccgcgcc cccgccgtgg cccgcccgct gagccgcgc
241 241 tgagcgcgatg ggcctcgccg ggcggggagc cagggccccg gccgcgcctc cacagccgcc
301 301 gcgcgctgga gagattgatt ccttggggag ctgtaagtac tgaggattta ggggtgcagcg
361 361 ctcatgttct actgatgcag agtccccaaa tgaatgtcca ggagcagggt ttccccctgg
421 421 acctcgagc aagtttcacc gaagatgccc ccggcccccc agtgccctgga gaagaggag
481 481 aactggatc tactgattcg aggcctgtca accacagttt ctgttctggg aaaggtacca
541 541 gcattaaaaa tgagacctca acagccacc caagacgttc agatctggat ctgggatatg
601 601 agcccgaggg cagtgcctcc ccacccccac catatttgag gtgggctgag tcactgcatt
661 661 ccttactgga tgaccaagat gggatcagcc tgttcaggac ttctctgaag caggagggct
721 721 gtgctgacct gctggacttc tggtttgctt gcagtggctt caggaagctt gagccctgtg
781 781 actcaaatga gaaaagagg ctgaagctgg caagagccat ctaccgaaag tacatcctgg
841 841 atagcaatgg catttgttcc agacaaaacca agccagccac taagagcttc ataaaggact
901 901 gtgtcatgaa gcagcagata gatcctgcca tgtttgacca ggcacagaca gaaatccagt
961 961 ccaccatgga ggagaatacc tacccttctt ttcttaagtc tgacatttat ttggagttaca
1021 1021 caaggacagg ctacagagat ccgaaggctt gcagtgaacca gagctcaggg tctggaacag
1081 1081 ggaaggggcat gtctggatat ctgcccactt tgaatgagga tgaagaatgg aatgtgacc
1141 1141 aagatgcaga tgaggatgat gcccagagacc ctctcccccc cagcaggctc accagaagc
1201 1201 tgctattgga gactgctgcc ccgaggggccc cctcaagtag acggtacaac gaaggcagag
1261 1261 agctcaggta tggatcttgg agggagccccg tcaaccctta ctacgtcaac tctggctatg
1321 1321 ccctggcccc agccaccagt gccaatgaca gtgagcagca gagcctgtcc agtgatgctg
1381 1381 acacgctatc ccttacggac agtagtgtgg atggaatccc ccatacagg atccgtaagc
1441 1441 agcaccgaag ggagatgcag gagagtatcc aagtcaatgg gcgggtacct ctacctaca
1501 1501 ttcttcgcac ttaccgaatg ccaaggaga tccgggtaga gccacagaaa ttgtctgaag
1561 1561 agcttattca ccgtctagag gctgtccagc gactcgaga ggctgaagaa aagtggagg
1621 1621 aacggctgaa gcgtgtacgc atggaggaaag aagggaggga tgggtgaaatg ccttctggcc
1681 1681 ccatggcaag tcacaagctg ccttctgtcc cagcttggca ccatctccca cccgctatg
1741 1741 tggatatggg ctgctcttga ctgctgggatg cccatgagga gaatcctgag agcatcctgg
1801 1801 atgagcacgt gcaagggtc atgaggacac ctggctgcca gtacacctggc ccaggccacc
1861 1861 gctctcctga cagtggggcat gtggctaaaga ctgcagtgtc aggggggtaca gcctccgggc

16/19

FIG. 9B

1921 atggaagca tgttcctaag ttaggggtga agctggatag agctggcctg caccatcata
 1981 gacatgtcca ccaccatgtt caccataatt cagctagacc taaggagcaa atggaggctg
 2041 aagttgcccg cagggtccag agcagcttct cgtggggccc agaacaacat ggtcatgcca
 2101 agccccggag ctattccgag aacgcaggca ccaccctcag tgcctgggat ttgccccttg
 2161 gtggtaaaaa tagtgcacct tccaaaagaa acaccaagaa ggcctgaatct gggaagaatg
 2221 ccaatgctga ggtacccagt accacagagg acgctgagaa gaaccagaag atcatgcagt
 2281 ggaatcattga gggagagaag gagatcagta gacaccggaa ggcaggccat gggctctctg
 2341 ggttgaggaa gcagcaggcc catgaaagct ccaggccctt gtccatcgag cgtccctggg
 2401 ccgtgcaccc ctgggtcagc gctcagcttc ggaattctgt ccagccttct catcttttca
 2461 tccaagatcc cacaatgcca ccaatccag cccctaattcc cctgacccag ctggaagagg
 2521 ccgcaggcg tttggaagaa gaagaaaaga gagcaacaa actggccctcc aagcagaggt
 2581 atgtgcaggc agtcatgcag cggggacgca cctgtgtcag gacagagaca aatcacaaa
 2641 tgagtgtggt accagccgtg tcggacttg aactctcga aactgtcag gtgtgtggc tactatttct
 2701 gaaaggcagg tggcgggagt gcaccacct gacacagcat tgtagggccg tgcctgtcct
 2761 gtggggaacc catccctac cggaccctgg tgaggggccg acagatacta cttaagaaa
 2821 tcaaggagct gctaaccaag aaggggagct tacgggagg aagctggactg agcactggg
 2881 agtttgactg tgggtgtgta gatcatcggc gcattgtcat cagttaggtg gacagccttg
 2941 ttgaagaaaa gatcatcggc tcaactactg gaaacaacac aagattgtgt catgagctct
 3001 gagcacacca cctgggtgtg gaaacaacac aagattgtgt catgagctct tctatcggg
 3061 cctgggtgtg gaaacaacac aagattgtgt catgagctct tctatcggg tgaagcttgg
 3121 gaccttaggt gtctgccagc ctctgtccct ctggctttgg gaaagtgggg gtgggggggtc
 3181 gtcctactga gtggttctct ggttttctct gttttctctgt tcaagaggaa agtccactt
 3241 accaccacat taccctctga accccctga agcaatacca ggagccatct catgacccctc
 3301 gcttctgaat ccagctctgac ctgggacctt tccactgcac ttggcccttg gcttgtatcc
 3361 ttctctctct tccctgtggg gcctctatat attgtacatg tccactgagt ccttcaacat
 3421 tccctgtggg gcctctatat attgtacatg tccactgagt ccttcaacat agctgtctct
 3481 tgcctgccac tgtgtgaatc tggcagctga gtatctcagg cccctttgcc tgtctccagc
 3541 caccagcttg gtctcagcagg agggggggcg gtgtgtcttg tcccttccaa tgtgtccgtgt
 3601 aaatatgtac atttctcagg ccaggggccag caggggggata cctgagccc attttctcatg
 3661 caatgacttg tacaattatc ttttcaagg tacttggata ataataaat aaaaacgttt
 3721 ttgaaccttc caaaaaaaa aaaaaaaa a

17/19

FIG. 10A

```

1  gggccccggg  tccgccacc  accgcgcgcg  ggacagattg  attcaacttg  gagctgtaag
61  tactgatgta  tragggtgca  gcgctcattg  ttcattgacg  cagagtccca  aatgaatat
121 ccaagagcag  ggtttccct  tggacctcgg  agcaagtcc  accgaagatg  ctccccgacc
181 cccagtgcct  ggtgaggagg  gagaactgg  gtccacagac  ccgaggcccc  ccagctacag
241 tttctgctcc  gggaaagggt  ttggcattaa  aggtgagact  tcgacggcca  ctccgaggcg
301 ctccgatctg  gacctgggt  atgagcctga  gggcagtgc  tccccaccc  caccatactt
361 gaagtgggt  ggtcactgc  attccctgct  ggatgacca  gatgggataa  gcctgttcag
421 gactttcctg  aagcaggagg  gctgtgccga  cttgctggac  tcttggtttg  cctgcactgg
481 cttcaggagg  ctggagccct  gtgactcgaa  cgaggagaag  aggtgaagc  tggcgagagc
541 catctaccga  agtacattc  ttgataacaa  tggcatcgtg  tccgggcaga  ccaagccagc
601 caccagagc  ttcataaagg  gctgcatcat  gaagcagctg  atcgatcctg  ccatgtttga
661 ccaggccccg  accgaatcc  aggcactat  ggaagaaaac  acctatccct  ccttccttaa
721 gtctgatatc  tatttggaa  atacgaggac  aggtcggag  agcccaag  tctgtagtga
781 ccagagctct  ggttcaggga  cagggaagg  catacttga  tactgcca  cctbaaatga
841 agatgaggaa  tggagtgtg  accaggacat  ggacgaggac  gatggcagag  acgtgtctcc
901 ccccggaaga  ctccctcaga  agctgtcct  ggagacagt  gccccgagg  tctcctccag
961 taagcgtac  agcgaaggca  gaggttcag  gtaggatacc  tggcgggag  cagtcacccc
1021 ctattatgtc  aatjccggct  atgccctggc  cccagccacc  agtgccaacg  acagcgagca
1081 gcagagcctg  tccagcgatg  cagacacct  gtccctcacg  gacagcagcg  tggatgggat
1141 ccccccatc  aggatccgta  agcagcacc  caggagatg  caggagagcg  cgcagggtcaa
1201 tgggcgggtg  ccctacctc  aggtcccg  caggtaccg  gtgccgaag  aggtccgct
1261 gagcctcag  aagtccgcg  aggagctcat  ccacggcctg  gaggtgtgc  agcgacgcg
1321 gagggccgag  gagaagctg  aggagcgct  gaagcgctg  cgcattggag  aggaaggta
1381 gagcggcgat  ccctgctcag  gggccccagg  gccgttcac  aagtgccctc  ccgccccgc
1441 tggcaccac  tccccccc  gcttgtgtg  gacatgggt  tbtgccggg  tccjggatgc
1501 acacgaggag  aacctgaga  gcctcctgga  cgagcagcta  cagcgtgtgc  tgaggacaac
1561 tggccgccag  tcgctgggc  ctggccatcg  ctccccggac  agtggggcac  tggccaagat

```


18/19

FIG. 10B

```

1621 gccagtggca ctgggggggtg ccgcttcggg gcacggggaag cactatccca agtcaggggc
1681 gaagctggac gcggccggcc tgcaccacca ccgacacgtc caccaccacg tccaccacag
1741 cacagcccgg cccaaggagc ccaaggagc aggtggaggg cgagccacc cgagggccc agagcagctt
1801 cgcctggggc ctggaaccac acagccatgg ggcaaggcc cgaggtact cagagagtg
1861 tggcgctgcc cccaacgcca gtgatggcct cgccacagt gggaagtg gggtgcgtg
1921 caaagaaat gccaaagag ctgagtcggg gaagagcgc agcaccgag tgcagggtg
1981 ctcgaggat gcggagaaga accagaaat catgcagtg atcattgag gggaagga
2041 gatcagcagg caccgcagg cggccacgg gtcttcggg acgaggaag cacagccca
2101 tgagaactcc agaccytgt ccttcgagca cccctggcc ggccctcag tccggacctc
2161 cgtgcagccc tccacctct tcatccaaga cccaccatg caccaccac cagctccca
2221 cccctaacc cagctggagg aggcgcggc acgtctggag gaggaagaa agagagccag
2281 ccgagcacc tccaagcaga ggtatgtgca ggaggtcttg cggcggggac gcgcctgcg
2341 caggccagc tgcgcgcgg tctgcacgt ggtaaccgcc agtcgggaca tggagctctc
2401 cgagacagag acaagatgc agaggaaggt ggccggcgg agtgcaccg cgtgtgacag
2461 catcgttggt gcgtactact tctgcgggga accatcccc taccgaccc tggtagaggg
2521 ccgcgtgtc accctggcc agttcaagg gctgctgacc aaaagggca gctacagata
2581 ctacttcaag aaagtgagc acgagtttga ctgtgggtg gtgttgagg aggttcgaga
2641 ggacgaggcc gtcctgccc tcttcgagg gaadacatc ggcaaatgg agaaggtgga
2701 ctgataggct ggtgggctgg ccgctgtgcc afgcgaggcc ctggcgggc acgggtgtca
2761 cggccaggca gatgacctg tactcaggag ccgatgggg aacagtgtg ggtgtaccac
2821 ccattccctgt ggtctacccg tgtctagagg caggtagggg gtccctccaa gtgtccaca
2881 agcttctgtc ctgcccccaa ggagcgagcc tggaccactc ctcatagcaa tacttgagg
2941 gccagccca agtgaggcag ccgaggtccc tjtgcaccg ttcaggtag cccccccat
3001 ccccgccac ctccctggg cactgtgtct ggatctact tccctctgg gatttgccca
3061 cgtacccagg tctggctggg gccaggccc ggtatgcag ggctgcagg cctctgcaa
3121 ttgtacgcgc caccagtgc cttcaacaca gcttgtctt tgcctgccac tgtgtgaatc
3181 ggcagcggag cactgcacct gcctccagcc gccgcttgg cagtcccttc tccctcttc
3241 tgaggggccc tgtaaatatg tacatttctc aggttagggc cagcaggggc tggccagtc
3301 tgtttttcat gcgatgacac ttgtacaatt atctttcaa aggtacttgg ataataatga
3361 aataaaactg ttttgaacc tgaataaaa aaaaaaaaaa a

```

19/19

FIG. 11

/translation="GPGSRHHRARDRLIHFGAVSTDLGCSAHCSLTQSPKMNIEQG
FPLDLGASFTEDAPRPPVPGEEGELVSTDPSPASYSFCSGKGVGKGETSTATPRRS
LDLGYEPGASPTPPYLKWAESLSLLDDQDGISLFRFLKQEGCADLLDFWFACTG
FRKLEPCDSNEEKRLKLARAIYRKYILDNNGIVSRQTKPATKSEIKGCIMKQLIDPAM
FDQAQTEIQATMEENTYPSFLKSDIYLEYTRTGSESPKVCSDQSSSGTGKGISGYLP
TLNEDEEWKCQDMDEDDGRDAAPPGRLPQKLLLETAAPRVSSRRYSEGREFRYGSW
REPVPYVYNAGYALAPATSANDSEQQLSSDADTLSLTDSVVGIPPYRIRKQHRRE
MQESAQVNGRVPPLPHIPRTYRVPKVVRVEPQKFAEELIHRLEAVQRTREAEKLEERL
KRVRMEEEGEDGDPSSGPPGCHKLPPAPAWHHEPPRLCWTWACAGLRDAHEENFESI
LDEHVQRVLRITGRQSPGPGHRSPPDSGHVAKMPVALGGAASGHGKHVPKSGAKLDAAG
LHHHRHVHHVHHSTARPKEQVEAEATRRRAQSSFAWGLEPHSHGARSRGYSESVGAAP
NASDGLAHSGKVGACRKNACKAESGKSASTEVPGASEDAEKNQKIMQWIIIEGEKEIS
RHRRGTGHSSGTRKPPQHENSRLSLEHPWAGPQLRTSVQPSHLFIQDPTMPHPAPN
PLTQLEEARRRLEEEKRAASRAPSKQRYVQEVNRRGPACVRPACAPVLHVVPVAVSDME
LSETETRSQRKVGGGSAQPCDSIVVAYYFCGEPIPYRTLVRGRAVTLGQFKELLTKKG
SYRYYFKV'SDEFDGCVVFEVREDEAVLPVEEKIIGKVEKVD"

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14414

A. CLASSIFICATION OF SUBJECT MATTER														
IPC(6) :Please See Extra Sheet.														
US CL :Please See Extra Sheet.														
According to International Patent Classification (IPC) or to both national classification and IPC														
B. FIELDS SEARCHED														
Minimum documentation searched (classification system followed by classification symbols)														
U.S. : 435/6, 69.1, 320.1, 325; 514/2, 44; 536/23.1, 23.5, 24.5, 24.31; 530/350; 800/2														
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched														
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)														
Please See Extra Sheet.														
C. DOCUMENTS CONSIDERED TO BE RELEVANT														
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.												
X,P ----- Y,P	Zeng, L. et al., The Mouse Fused Locus Encodes Axin, an Inhibitor of the Wnt Signaling Pathway That Regulates Embryonic Axis Formation. Cell. 11 July 1997, Vol. 90. pages 181-192, especially page 184, Figure 3, page 186, "The Axin Gene," 1st and 2nd paragraphs and page 190, "Experimental Procedures," entire section.	1-15 ----- 16-84, 90-92												
Y	Fujimoto, J. et al. Progestins and Danazol Effect on Cell-to-cell Adhesion, and E-Cadherin and alpha- and beta- Catenin mRNA Expressions. J. Steroid Biochem. Molec Biol. 1996 Vol. 57. No. 5-6. pages 275-282, especially page 278, Figure 6.	93, 96												
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.														
<table border="0"> <tr> <td>* Special categories of cited documents:</td> <td>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>*A* document defining the general state of the art which is not considered to be of particular relevance</td> <td>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>*B* earlier document published on or after the international filing date</td> <td>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>*A* document member of the same patent family</td> </tr> <tr> <td>*O* document referring to an oral disclosure, use, exhibition or other means</td> <td></td> </tr> <tr> <td>*P* document published prior to the international filing date but later than the priority date claimed</td> <td></td> </tr> </table>			* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family	*O* document referring to an oral disclosure, use, exhibition or other means		*P* document published prior to the international filing date but later than the priority date claimed	
* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention													
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone													
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art													
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family													
O document referring to an oral disclosure, use, exhibition or other means														
P document published prior to the international filing date but later than the priority date claimed														
Date of the actual completion of the international search		Date of mailing of the international search report												
29 SEPTEMBER 1998		26 OCT 1998												
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231		Authorized officer PETER TUNG												
Facsimile No. (703) 305-3230		Telephone No. (703) 308-0196												

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/14414

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 38/17, 48/00; C07H 21/04; C07K 14/435; C12N 15/00, 15/63, 15/85; C12Q 1/68

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

435/6, 69.1, 320.1, 325; 514/2, 44; 536/23.1, 23.5, 24.5, 24.31; 530/350; 800/2

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, CANCERLIT, CAPLUS, DISSABS, BIOSIS, EMBASE, WPIDS

SEARCH TERMS: Axin, FU gene, Fused, beta catenin, mouse, locus, therapy, Frank Constantini, Frank Costantini, Li Zheng